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Work package 1 report: Hazard identification for vertical transfer of fish disease agents

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English summary:
Focus of this report has been to establish the evidence – or lack of such – that vertical transmission of selected fish diseases does actually occur and in particular whether transmission may occur inside the fertilised egg ("true vertical transmission") or only as contamination of the egg surface ("egg-associated transmission"). The results show that there is scientific evidence for true vertical transmission of BKD, IPN and piscirickettsiosis, whereas experimental and field studies suggest that vertical transmission of VHS, IHN and ISA may be effectively prevented by common egg disinfection procedures. Flavobacteriosis is believed vertically transmissible even though attempts to provovide defintive evidence experimentally have failed. For several of the diseases addressed, there is a remarkable lack of published scientific information. A priority list of research topics to clarify the hazard potential of these diseases is being proposed.

Norsk sammendrag:
Denne rapportens hoverfokus har vært å vurdere de vitenskapelige bevis (eller mangel på sådan) som finnes for at definerte fiske-sykommer faktisk kan overføres vertikalt, dvs. fra for foreldrefisk til avkom. Særlig har arbeidet tatt sikte på å avklare om det forekommer "sann" vertikal overføring (dvs. embryonal infeksjon) eller om overføring kun skjer ved utvendig kontaminering. Resultatene viser at BKD, IPN og piscirickettsiose kan smitte ved "sann" vertikal overføring, mens VHS, IHN og ILA synes å kunne forebygges effektivt ved vanlige desinfeksjonprosedyrer. Flavobakteriose antas å smitte vertikalt selv om forsøk på å bevise dette eksperimentelt har mislykkes gjentatte ganger. For flere av sykdommene som er undersøkt finnes det ikke nevneverdig publisert informasjon om emnet. En prioritert liste over framtidige forskningsemner foreslås.

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Introduction

National and international trade in fertilised eggs and gametes for finfish aquaculture is in most parts of the world subject to strict zoo-sanitary regulations and certification requirements, many of which are built upon rather old and partly scarce scientific data. Aim of this concerted action project is therefore to scrutinise and re-assess the scientific basis for current zoo-sanitary control requirements. Focus of this work package has been to establish the evidence – or lack of such – that vertical transmission does actually occur, and in particular whether transmission may occur inside the fertilised egg ("true vertical transmission") or only as contamination of the egg surface ("egg-associated transmission").

Material and Methods

Based upon the participanting scientists' background knowledge, and the study of international recommendations as well as European and national legislation, a number of fish diseases were selected by the project group for in-depth study and full scientific review (attachment 1). For each of these diseases, a comprehensive collection of both published studies and written, but unpublished information was prepared, and external experts were consulted. Three working meetings with invited external experts where conducted by the project group in the preparation of this report. During these meetings, the interpretation of scientific data and the the conclusions to be drawn for the report were discussed and agreed upon. Draft manuscripts for various diseases were submitted and edited into a draft report, which has been distributed the contributing experts for supplementation and revision. The work, including further improvement of the initial version of the report has been co-ordinated by Instituto Zooprofilattico delle Venezie, who has been the co-ordinator of this report.

Results

Rhabdovirus infections
Infectious haematopoietic necrosis

Brief disease description

Historically, the geographical range of infectious hematopoietic necrosis was limited to the western parts of North America, but the disease, caused by a rhabdovirus (IHNV), is now also present in continental Europe and the Far East. The principal clinical and economic consequences of IHN occur at farms rearing rainbow trout in freshwater; however, both Pacific and Atlantic salmon can be severely affected. Rainbow trout, steelhead and Pacific salmon are only affected in freshwater environments and susceptibility to the virus generally decreases with increasing fish size and age. However, Atlantic salmon can be severely affected in both freshwater and marine life stages and susceptibility does not appear to be effected by the size or the age of the fish. Mortalities due to IHN have also been recorded among some wild stocks of salmonids. The most prominent environmental factor affecting IHN is water temperature. Clinical disease occurs between 8°C and 15°C under natural conditions. Infection is often lethal due to the impairment of osmotic balance, and occurs within a clinical context of oedema and haemorrhages. High levels of virus are shed from infected juvenile fish. Adult fish are generally asymptomatic carriers that may shed virus in their sexual products at the time of spawning. Survivors of IHNV infection demonstrate a strong protective immunity with synthesis of circulating antibodies to the virus and, in certain individuals, a covert carrier state.

Review of evidence for vertical transmission

In the literature pertaining to IHN virus, "vertical transmission" is often referred to as the transmission of virus from the parent fish to its offspring, regardless of whether the virus is located inside or outside of the egg (Pilcher and Fryer, 1980a,b). This makes the assessment of the risk for true vertical or intra-ovum transmission of IHNV to occur difficult to determine from the literature. 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 detected before and the 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 most likely is a very infrequent event. There are several reports where IHNV-infected parents 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 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 and fry of infected adult sockeye salmon. They found that only a small portion of sockeye eggs and fry contained IHNV and that not all parents transmitted the virus to their progeny and 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 within some groups of fish at only some aquaculture facilities. Alternatively there are numerous other variables that are unknown and/or uncontrollable that could initiate an IHN epizootic intermittently in a susceptible group of fish.

Reproductive fluids from infected fish can have detectable IHNV, but it is controversial whether the virus is only on the egg surface or within the egg. When eggs from masu salmon and chum salmon were exposed to IHNV and then fertilized, the eggs and resulting fry were not infected (Yoshimizu et al. 1989). The stage of egg development also affected the ability of the virus to replicate. Exposure of unfertilized eggs to virus did not result in viral replication, but when eyed eggs were injected with IHNV, the virus replicated and the resulting fry suffered IHN-induced mortality. Egg-yolk components inhibited viral replication. The ability of IHNV to replicate after injection into the egg appeared to correlate with increased embryo development and a decrease in yolk components. Yoshimizu et al. (1989) also concluded that the risk of true vertical transmission of IHNV occurring is doubtful.

Infectious hematopoietic necrosis virus is frequently isolated from milt (Wingfield and Chan, 1970; Meyers et al., 1990; Yamazaki and Motonishi, 1992) and male salmonid fish may have a role in vertical transmission. Sockeye salmon and steelhead trout have a lower prevalence of infection and viral titres in milt than in the kidney and spleen (Mulcahy et al., 1987). The prevalence of infection in milt is lower than that in ovarian fluid, but the proportion of males with high milt viral titres may be equivalent to that of females with high virus concentrations in the ovarian fluid (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 which has adsorbed IHNV from 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 is still 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.

Experiences in the laboratory and in the field evaluating the risk of true vertical transmission led to the conclusion that the risk is negligible. Primary management strategies for minimizing this risk are to use a 100 ppm iodophor solution for disinfection of the egg during waterhardening, after fertilization of the egg, and also at the eyed-egg stage just prior to movement and hatching. Additionally, all egg incubation and early life stage fish rearing (up to ~ 4 g) should be done in virus-free water supplies (LaPatra, pers.comm.).

Priority research needs regarding hazard identification

1. Extensively test surface sterilized green and fertilized eggs from high titered IHNV-positive adults using highly sensitive detection techniques such as cell culture, nested PCR and real-time PCR.

2. Examine ova from different trout and salmon species exposed to different IHNV strains at intervals during incubation using the techniques above to further assess potential hazards.

3. Assess the survivability of IHNV in yolk components obtained from different species using an in-vitro assay.

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Viral haemorrhagic septicaemia

Disease description

Viral haemorrhagic septicaemia (VHS) is an infectious disease of rainbow trout *(Oncorhynchus mykiss)* which may also affect brown trout *(Salmo trutta),* grayling *(Thymallus thymallus),* whitefish (*Coregonus* spp.), pike *(Esox lucius)* and turbot *(Scophthalmus maximus).* It is caused by generalised infection with VHS virus (synonym: Egtved virus), a virus belonging to the genus Novirhabdovirus within the family Rhabdoviridae. Clinical signs of acute infection are oedema, haemmorhages and osmotic imbalance.

Until the mid-1980s, VHSV was only believed to affect rainbow trout and a few other cultured freshwater fish species in continental Europe. In the last decade, however, VHSV has been isolated from a large range of free-living marine fish species in the North American part of the Pacific Ocean, in the North Atlantic and the Baltic Sea, some of which are also associated with epizootics of clinical disease. Whereas various isolates are serologically indistinguishable, genetic analyses show that isolates from freshwater fish are relatively homogenous as compared to the marine isolates. Marine isolates tested experimentally have shown no or only low virulence to rainbow trout fry.

Water temperature is an important environmental factor in VHS as overt disease only occurs at temperatures between ca. 4°C (protracted course) and ca. 14°C (acute course of infection). In endemic areas, infected fish become silent carriers of the infection during winter, allowing for the seasonal cycle of infection to be completed within the susceptible population. During acute disease, virus is massively shed through faeces, urine, skin and gills.

Review of evidence for vertical transmission

In a few early papers on VHS, speculations were raised that the occurrence of the disease in rainbow trout fry and the apparent spread of VHS was due to vertical transmission (Ghittino 1965, Håstein et al. 1968). Ghittino reported of VHSV infection in a hatchery without any previous history of VHS, where the affected group of fingerlings originated from eyed eggs imported from another hatchery with endemic VHS. At that time Jensen (1965) had shown that virus could be propagated in primary cell cultures prepared from immature rainbow trout ovaries.

The first experimental study on vertical transfer of VHSV was, however, published by Vestergård Jörgensen (1970) who used non-disinfected, fertilised eggs originating from latently infected rainbow trout for transmission experiments. Immediately after fertilisation, eggs where found virus-positive by cell culture. One group of eggs was then bath exposed to cell culture grown Egtved virus whereas another group remained unexposed. Both groups where transfered to hatching cylinders with circulating, VHSV-free water. The naturally infected eggs remained virus-positive for 3.5 hours only, whereas the experimentally infected eggs where virus-positive until day 10 but not later. It was not possible to isolate VHSV from either group of fry post-hatching. Referring also to unpublished field observations, the author concluded that VHS virus may be present as contaminant on the egg surface, but that it apparently does not survive the egg incubation period. This was also the first report on VHSV isolation from fish eggs.

The adsorption of salmonid viruses to fish sperm was studied by Mulcahy and Pascho (1984). These authors were unable to demonstrate absorbtion of VHSV to chinook salmon sperm, whereas another rhabdovirus (IHNV) adsorbed readily and infectious pancreatic necrosis virus (IPNV) showed some adsorption. To our best knowledge, there is no scientific report demonstrating the presence of VHSV in fish sperm although it appears possible that contamination of the sperm may occur when stripping latently infected, mature rainbow trout males.

Both Vestergåard Jørgensen (1973) and other authors (Amend and Pietsch, 1972) found that VHSV was rapidly inactivated by iodine compounds recommended for disinfection of salmonid eggs. Together with studies showing that VHSV has a limited survival in water, and is highly susceptible to ultraviolet (UV) light (Ahne 1982), this may explain why there are no later reports implicating egg-associated transmission of VHSV (Wolf 1988).

Priority research needs regarding hazard identification

We suggest that research to clarify if VHS (if at all) can be vertically transmitted should focus on the following tasks:

1. Investigation on the presence of VHSV inside eggs from latently infected motherfish and from experimentally exposed eggs, using currently available molecular genetic methods (PCR).

2. Studies on the attachment of VHSV to rainbow trout sperm by use of various methods, such as immuno-gold staining and virus capture assays like those employed by Mulcahy & Pascho (1984).

3. Evaluation of the effects of cryopreservation on VHSV survival in contaminated sperm samples.

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Spring viraemia of carp

Disease description

Spring viraemia of carp (SVC) is an acute disease of common carp *(Cyprinus carpio)* and other cyprinid species that is caused by a systemic infection with a piscine rhabdovirus *(Rhabdovirus carpio).* Clinical manifestation of the infection occurs mainly in their first and second year of rearing. The disease is prevalent in many countries of continental Europe where common carp is abundant, and which experience cold winter temperatures. Outbreaks of SVC in ornamental (koi) carp were recently for the first time reported in Germany (Neukirch & Kunz 2001) and in USA (anonymous 2002).

The main reservoirs of SVCV are covert virus carriers present in cultured or natural populations and the main route of transfer is horizontal, via live fish movements or through virus shed with faeces, urine, mucus, or exsudates. In the epidemiology of the disease, a seasonal circulation of SVCV is postulated. In several cases, SVC virus was isolated from carp experiencing sub-acute infection during the autumn months (Osadchaya et al., 1982; Shchelkunov & Shchelkunova, 1990). The experimental findings of A.-M. Baudouy et al. (1980), suggest that transformation of autumnal sub-acute to over-winter chronic SVC infection is possible, eventually resulting in acute disease outbreak in springtime. Recently, Shchelkunov et al. (1999a,b) showed experimentally by modeeling seasonal temperature conditions that SVC virus can survive in carp over the whole summer, autumn and winter seasons to cause infection in naïve fish during the low-temperature season. This work supports that a population of carp, *Cyprinus carpio,* may be self-sustaining for year-round maintenance of SVCV infection. A major biological bottleneck for both horizontal and vertical transmission of SVCV is the summer season of high water temperatures that threaten virus survival in its principal host.

Review of evidence for vertical transmission

Because of very short time interval between egg fertilization and hatching, trade and transfer of carp takes place at the larval or early fry stages, whereas gametes or fertilised eggs are normally not transferred between fish farms or watercourses. The fact that maturation and spawning in the carp normally takes place at water temperatures that are prohibitive for SVCV replication in immunophysiologically normal spawners suggests that vertical transfer of SVCV is unlikely. After gonadotropic injection is given to overwintered carp, the fish must be kept for not less than one week at water temperature of above 18 °C before its maturation is completed and successful egg stripping can be performed. According to W. Ahne (1979, 1986), this time interval was sufficient for 750 g carp held at 15-20 °C to clear themselves of the virus. In addition, we have not been able to find reports of natural SVC outbreaks in carp larvae or very young fry, that might be suggestive of vertical SVCV transmission. The only case report by N. Fijan et al. (1981) on SVCV-caused disease outbreak in 7-day old sheatfish fry, *Silurus glanis,* in a water recirculation system is considered an illustration of the SVCV capability to laterally infect a quite broad spectrum of very young susceptible hosts, rather than of the true vertical transfer of the virus. In fact, there is no direct evidence available to date that could be supportive of vertical SVC virus transmission in carp.

To assess the problem from a theoretical angle, the reproduction and growth cycle of carp can be broken down to several phases, wich will be discussed separately.

Carp spawners: There are enough data reliably demonstrating that SVC virus can cause acute disease in carp spawners. Amongst the field data, Osadchaya (1977) and Osadchaya et al. (1982) reported on several virus isolations from diseased broodstock fish in spring time. Outbreaks of SVC in adult 30-60 cm long carp occurred in spring 1991 in ponds located in Central Spain (Marcotegui et al., 1992). Under experimental conditions, W. Ahne (1979) reported on mortality of artificially infected adult (750 g) carp held at 10^{0}C and Shchelkunov et al. (1993) registered 60% morbidity and 50% mortality with virus titres up to 107 – 109 per gram tissue in mature 3-yr-old carp (0.65 – 1.0 kg body weight) held at 12.5-17.5^{0}C after i.p. injection of the virus.

Spawners' sexual products: Unfortunately, relevant information on the presence or absence of SVCV in sexual products is scarce. Bekesi and Csontos (1985) succeeded in isolating SVCV from only 3 ovarian fluid samples out of 491 tested, and from none of 211 seminal fluids sampled from hypophysed 5-10-year-old carp. The study was conducted in May, at water temperatures between 10-18^{0}C. These data suggest that virus infection of carp ovarian fluid is a rather rare event. However, the authors provided no comments upon whether all of the fish farms surveyed were located in a naturally

infected zone. Unfortunately, samples of the examined sex products were obtained by fish stripping – the technique that does not exclude a possibility of sample contamination with external virus (from fish feces, urine, mucus, etc). N. Fijan (1988) isolated no virus from sexual products of 50 females and 36 males from SVC-infected farms. Health status of the fish and water temperature were, however, not mentioned in the report. In a limited study, W. Ahne (1979, 1983, 1985) failed to re-isolate the virus from seminal fluid of carp spawners recovered after experimental SVCV infection.

Fertilized eggs and newly hatched larvae: The only relevant publication available is by W. Ahne (1979) who was unable to isolate virus from eyed eggs and larvae obtained from SVCV-free broodstock carp eggs fertilized with sperm of spawners recovered after experimental SVCV-infection. The number of fish examined was not given.

Very young fry: As mentioned above, no natural SVC outbreaks in carp fry have been reported despite the fact that very high susceptibility has been recorded in experimentally infected larvae and 3-4-wk-old fry at water temperature from 17-23^{0}C, indicated by losses from 60 to about 100% (Fijan, 1981; Hattenberger-Baudouy et al., 1987; Shchelkunov et al., 1993).

Based on the data presented above, our overall conclusion remains that vertical transmission of SVC virus has not been scientifically demonstrated and that such transmission (if at all possible) appears of minor epidemiological importance (Fijan, 1988; Ahne et al., 2002).

Priority research needs regarding hazard identification

Using common and/or koi carp as principal study species, we suggest that research should be focussed on the following tasks:

1. Development of robust methods for rapid, sensitive and specific detection and identification of SVC virus, and their application on sexual fluids and products. Presently existing monoclonal antibody based- and PCR-based techniques should be compared with the traditional virus isolation method.

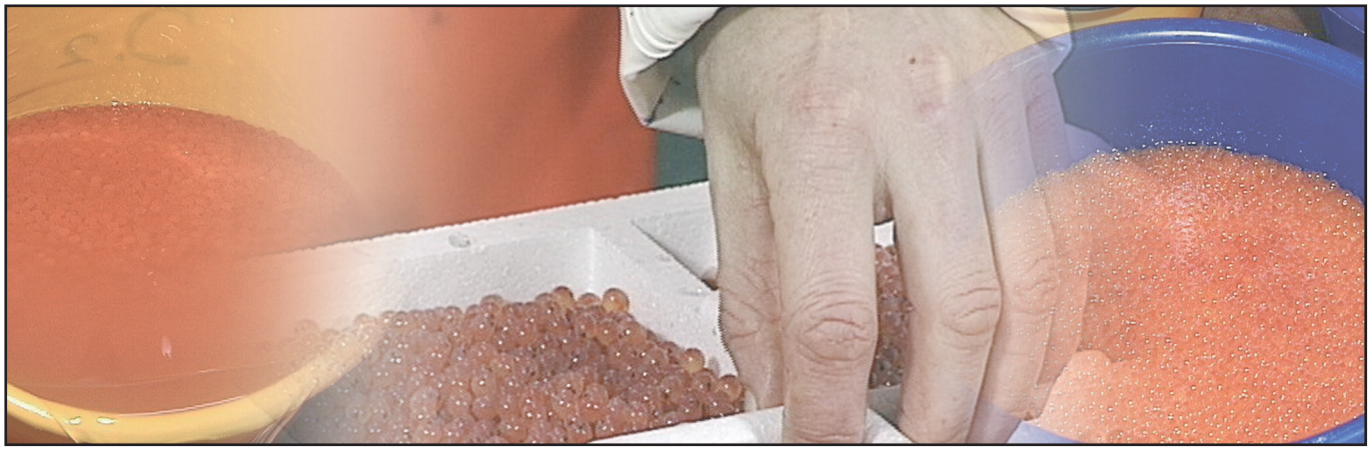
2. Observational studies attempting to identify and trace the fate of SVC virus along the reproduction chain from spawners' sex products to the very young fry. Investigations should be conducted in fish farms located in naturally infected zones.

3. Experimental studies using a) artificially virus infected spawners; b) sexual products artificially infected prior to fertilization; c) artificially infected eyed eggs. To confirm the presence of virus inside the egg or sperm cells, SVCV-positive samples could be additionally checked by using in situ techniques like fluorescent in situ hybridization (FISH) or similar.

4. The speed of virus clearance in latently infected spawners subjected to very rapid transfer from cold to warm water.

5. Evaluation of effects of cryopreservation on survival of SVC virus. This work should include investigation into the fate of SVCV in experimentally infected sperm stored in liquid nitrogen.

Pre-incubation of sexual samples at about 15^{0}C for 2-4 days before examination may be useful for in situ amplification of virus (Shchelkunov et al., 1999b).



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Iridovirus infections

Epizootic haematopoietic necrosis (including ESV and ECV infections)

Disease description

Epizootic haematopoietic necrosis (EHN) is caused by a systemic iridovirus (Ranavirus) infecting redfin perch *(Perca fluviatilis),* rainbow trout *(Oncorhynchus mykiss),* sheatfish *(Silurus glanis)* and catfish *(Ictalurus melas).* Three similar viruses associated with outbreaks of disease have been isolated and named epizootic haematopoietic necrosis virus (EHNV), European sheatfish virus (ESV) and European catfish virus (ECV). Although inducing similar diseases in their respective hosts, EHNV, ECV and ESV are presumably distinct agents distinguishable by molecular techniques. The disease caused by the three iridoviruses is characterised by mortalities due to necrosis in the liver, spleen, haematopoietic tissue of kidney and other tissues. The geographical range of EHNV is currently restricted to Australia, whereas the ECV and ESV agents have only been detected in Europe.

Review of evidence for vertical transmission

No published or unpublished data are available providing evidence either for or against vertical transmission of EHN or the presence of the causative virus(es) in the sexual products of susceptible host species. There are no field observations or other indirect evidence to indicate whether vertical transmission of EHNV, ESV or ECV may occur. The risk of vertical transmission is therefore impossible to assess at the present time. In view of the fact that these are extremely virulent viruses capable of causing mortality rates as high as 100% in farmed and wild populations, further research on the possibility of their transfer via eggs or sperm of infected broodfish is urgently needed.

Priority research needs regarding hazard identification

1. There is a need for EHN virus isolation to be conducted on gonad, milt or ovarian fluids of breeding perch in infected populations in Australia where the disease is endemic. Similar work is required for the ESV and ECV viruses in sheatfish and catfish, respectively.

2. Further research to characterise the viruses by molecular methods is needed to resolve the uncertainty as to whether ESV and/or ECV are true strains of EHNV or different viruses. Experimental challenge experiments with these two viruses in European perch would demonstrate whether they produce the same disease as EHN in perch from European populations.

References

No publications or other reports dealing with the issue of possible vertical transmission of EHN or on the detection of the causative virus(es) in gonads, sperm, ovarian fluid or eggs of susceptible host species have been found despite extensive searches of scientific literature databases and communications with external experts working on these viruses. In a personal communication, Professor Richard Whittington, the designated expert at the OIE Reference Laboratory for EHN, states that there are no records of EHNV having been recovered from gonads, sperm, ovarian fluid or eggs of affected European perch populations in Australia. It appears also that there are no records of tests for ESV or ECV viruses having been conducted on affected sheatfish or catfish populations in Europe.

Red sea bream iridoviral disease

Disease description

Red sea bream iridoviral disease (RSIVD) is a significant cause of mortality among cultured marine fish. The causative agent is the red sea bream iridovirus (RSIV).

The first outbreak of an RSIVD was recorded in cultured red sea bream in Shikoku Island, Japan in 1990. Since 1991, the disease has produced mass mortalities in cultured fish populations in the western part of Japan, mainly among juvenile red sea bream. However, mortality of market-sized fish has also been reported. Affected fish are lethargic, exhibit anemia and show enlargement of the spleen.

Review of evidence for vertical transmission

There is a total lack of evidence to review. No data have been published for or against vertical transmission of RSIVD. Also, personal communication with the OIE Reference Laboratory for the disease has not revealed any unpublished data relevant to vertical transmission.

Priority research needs regarding hazard identification

In view of the fact that there is no trade in the eggs or sperm of fish species known to be susceptible to RSIVD nor is any likely development of this seen, it is suggested that this disease may not be significant enough to be considered further in the FishEggTrade project.

References

No publications or other reports providing evidence for vertical transmission of RSIVD or the presence of the virus in gonadal tissues, eggs or sperm have been found despite extensive searches of scientific literature databases.

White sturgeon iridoviral disease

Disease description

The white sturgeon iridoviral disease (WSID) is a significant cause of mortality among farm-raised juvenile white sturgeon *(Acipenser transmontanus)* in North America and among Russian sturgeon *(A. guldenstadii)* in Europe.

The white sturegeon iridovirus is an epitheliotropic virus infecting the skin, gills, and upper alimentary tract. Infections of the oral mucosa and olfactory organ epithelium causes affected animals to stop feeding, leading to emaciation and starvation.

Review of evidence for vertical transmission

There is a severe lack of evidence for or against vertical transmission of WSIVD. The only relevant study published did not provide direct evidence of virus in the broodstock, eggs or sperm but a temporal/spatial statistical analysis of outbreaks in a white sturgeon hatchery in the USA led the authors to conclude that the observations supported a hypothesis of 'vertical transmission' of WSIV.

Priority research needs regarding hazard identification

The WSID virus is difficult to isolate in tissue culture and there is no currently available PCR method for its detection in fish tissues. Priority research need is therefore to develop virus detection methods in order to determine whether the virus is present in gonadal tissues, sperm or eggs before the risk of vertical transmission can be properly assessed.

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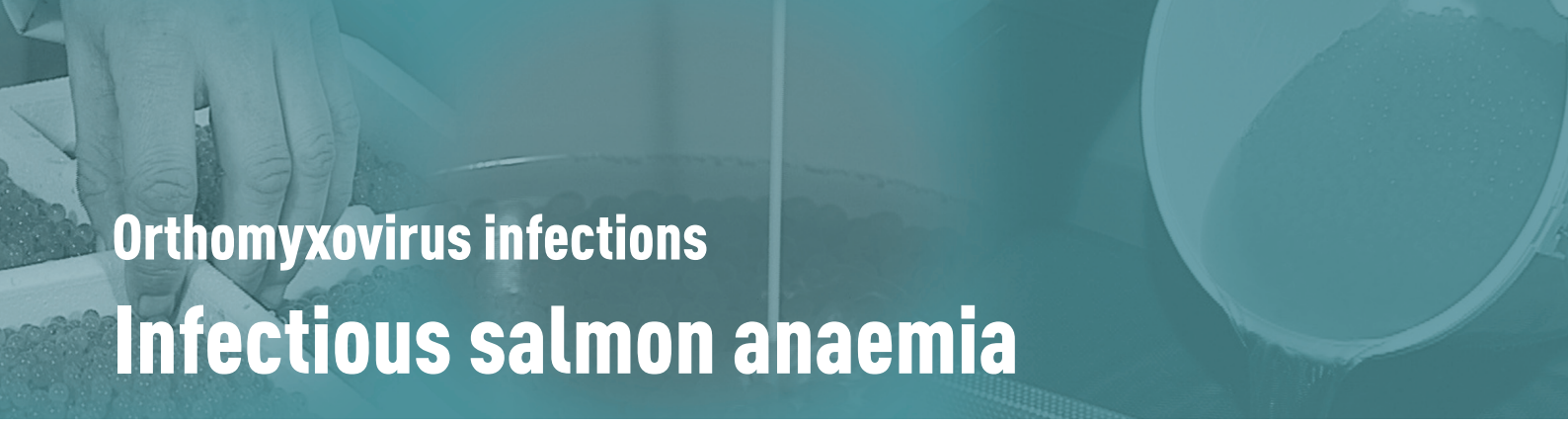

Orthomyxovirus infections

Infectious salmon anaemia

Disease description

Infectious salmon anaemia (ISA) is an acute to chronic disease of sea water reared Atlantic salmon *(Salmo salar),* which is caused by an orthomyxo-like virus replicating primarily in endothelial cells. Its clinical manifestation comprises circulatory damage with haemorrhages and haemolysis. Re-activation of the infection is believed to occur in latently infected fish approaching sexual maturity, and various forms of physical stress are believed to trigger activation of the infection and clinical outbreaks in populations harbouring virus carriers.

ISA of Atlantic salmon has so far been confirmed in Canada (New Brunswick and Nova Scotia), Norway, the Faroe Islands and the United Kingdom (in Scotland and the Shetlands). Atlantic salmon is the only susceptible fish species known to develop clinical disease, but the ISA virus (ISAV) may survive and replicate in sea trout *(Salmo truttae),* rainbow trout *(Oncorhynchus mykiss)* and Atlantic herring *(Clupea harengus),* which thus may act as carriers of the virus. In Chile, a condition called "icteric syndrome" of coho salmon is suggested attributable to a related virus, but without conclusive evidence being availble as of yet.

Observational evidence regarding vertical transmission

Nylund al. (1995) reported on finding of ISA virus in gonads of Atlantic salmon undergoing acute infection, but concluded that the primary target tissues for the virus seems to be the vascular endothelium. Since gonadal tissues have vascular endothelial lining, it seems plausible that the virus may be present and multiply in the reproductive organs of infected fish. In fish that are only temporary carriers of the virus, however, there is no information to suggest the deposition of virus in eggs or milt (Rimstad pers. comm.).

Referring to the disease- and population history of a particular Atlatic salmon broodstock, H.O. Djupvik (pers. comm.) is convinced that this population was already infected with ISA in 1986, as the fish were restocked to sea water after stripping. This egg producer delivered some 30% of disinfected Atlantic salmon eggs to the Norwegian market in the following season. In 1987, eyed eggs from the same farm were distributed throughout the whole country after double disinfection. Despite this, it has in retrospect not been possible to trace any spread of ISA via the trade of these eggs. According to Djupvik, the distribution pattern of ISA in Norway in this and the following period indicates that vertical transmission was not the cause of disease spreading. Trade in live smolts, often subclinically infected, was considered the primary source of infection resulting in secondary infection to adjacent farms. This is also the conclusion of the Regional Veterinary Officer for the County Hordaland & Sogn and Fjordane, who have registered no epidemiological indications as regards vertical spread of ISA by eggs (M. Binde, pers. comm.).

These statements are supported by several epidemiological studies indicating that ISA is mainly spread by infected live Atlantic salmon or other infected biological material, i.e. wastes, discharge from normal operations and slaughter (Djupvik et al. 1992, Vågsholm & al.,1994, Jarp 1999). Passive virus transmission through sea water is seen as an important route for the spread of ISA (Jarp 1999).

Nylund et al. (1999) described an outbreak of ISA during first feeding in Atlantic salmon fry from a Norwegian salmon hatchery. The presence of ISA virus was shown in cell cultures (ASK-cells) and the findings confirmed by the use of transmission electron microscopy (TEM). A total of 22 fish larvae were also examined for ISA by the use of RT-PCR and 6 larvae were shown to be positive. The possibility that this might be a case of vertical transmission is discussed without a definite conclusion. In extensive samplings on a later stage by the veterinary authorities, attempts to isolate ISAV from the same population failed (T. Håstein, pers. obs).

To the best of our knowledge, there has been no export of live fish or fish eggs from Norway to Canada and thus such an explanation cannot be used for a possible vertical transfer of ISA to Canada. Furthermore, according to Bouchard et al. (1999) and Blake et al. (1999), the North American isolates of ISA virus make a distinct genomic variant from the described Norwegian strains, suggesting at least several decades of separate evolutionary history.

Melville & Griffiths (1999) reported on the absence of vertical transmission of ISA from individually infected Atlantic salmon, using eggs collected from ISAV- positive grilse as determined by RT-PCR in their ovarian fluid. ISA virus was not detected in eyed eggs, alevins or parr

and no mortality occurred among fish injected with egg homogenates derived from ISA-positive fish.

Based on studies with disinfected and non-disinfected eggs from ISAV-positive brood fish of Atlantic salmon from the Magaguadavic river (1999), Dr G. Olivier (pers. comm.) believes that there is no indication on vertical transmission of ISA or that the risk of ISA being vertically transmitted is very low to extremely low. In trials carried out on offspring kept for more than one year ISA virus was never isolated, but RT-PCR tests from some of the progeny after hatching were positive, results which are currently being scrutinised for consistency.

After the discovery of ISA in Norway and until 1998 (Dr A. McVicar, pers. comm.), Chile imported millions of Atlantic salmon eggs without requirements that the eggs should be derived from ISA free brood stock. Despite this, ISA has not been reported in the progeny of Atlantic salmon in Chile. Information from Dr. Pedro A. Smith (pers. comm.) indicated that no attempts had been done in Chile to detect ISAV in ovarian fluid, seminal fluid, milt, ovarian- or milt tissues in connection with the ¨ haemolytic anaemia of coho salmon¨ described from Chile, and from which an ISA virus was reported (Kibenge et al., 2001). According to Cunningham et al. (2002), who studied the highly polymorphic region of the haemagglutinin gene of ISAV, the sequence from the Chilean isolate was identical to the sequence of a Canadian isolate. To which extent this finding may support the hypothesis of vertical transmission is disputed.

In the interim report of the Scottish joint government/industry working group on infectious salmon anaemia, January 1999, it is concluded that there has not been reported any incidences of ISA virus being vertically transmitted from parents to juveniles. As Scotland did not import live fish or live eggs of Atlantic salmon from Norway since the early seventies, the introduction of the ISA to Scotland cannot have occurred by eggs.

Experimental work on vertical transmission of ISAV

Before ISA virus was identified by means of isolation in cell culture, early transmission experiments were conducted in Norway (Thorud & Djupvik, 1988, Thorud 1989, Thorud 1991). Attempts to demonstrate vertical transmission using egg homogenates from brood fish from an ISA affected farm failed, leading the authors to conclude that spread of ISA by eggs was not likely to occur.

In conclusion, there is no hard evidence for the vertical transmission of ISAV neither as an egg-associated contamination nor as true vertical transmission inside the egg, and vertical transmission is obviously insignificant in the epidemiology of the infection. Whereas external contamination of gametes or embryos cannot be excluded, the true vertical transmission of ISAV appears unlikely as deemed from the information currently available.

Priority research needs regarding hazard identification

1. New studies on the occurrence of ISA virus in gonadal tissues from infected fish.

2. Further studies on the presence and abundance of ISA virus in the ovarian -and seminal fluids from infected broodfish, and on the egg surface before and after disinfection.

3. Investigations on the attachment of ISAV to sperm (both naturally and experimentally contaminated).

4. Experiments on the presence and survival of ISAV within the fertilised salmon egg.

The methods recommended for detection of ISAV as it appears in the OIE International Aquatic Animal Health Code should be used in order to demonstrate both live viral particles and remnants of ISA virus.

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Other virus infections

Viral encephalopathy and retinopathy/ Viral nervous necrosis

Disease description

Viral encephalopathy and retinopathy (VER), or viral nervous necrosis (VNN) has been reported as a serious disease of larval and juvenile marine fish that occurs almost world-wide except for Africa. Main fish species affected are sea bass, halibut and striped jack, and the agents in striped jack and sea bass have been characterised and placed in the family Nodaviridae.

The diseases are characterised by various neurological abnormalities, such as swimming disorders and vacuolation of the central nervous tissues. There are considerable variations in the age at which disease is first noted and the period over which mortality occurs. In general, the earlier the signs of disease occur, the more severe are disorders and lesions and the greater is the rate of mortality.

Review of evidence for vertical transmission

Observations carried out in Italy following the first appearance of VER during summer 1995 (G. Bovo, pers. obs) clearly suggested that horizontal transmission represented the main route of transmission of VER from infected to unexposed populations of sea bass *(Dicentrarchus labrax).* Juveniles surviving VER outbreaks during the larval stage may harbour the virus for a long period of time and become a potential source of infection for unexposed populations when moved from hatcheries to on-growing facilities. The information obtained from the field has later been confirmed through experimental transmission trials (Peducasse et al. 1999) conducted in sea bass by immersion or cohabitation. Furthermore, similar results have been observed with striped jack *(Pseudocaranx dentex)* by Arimoto et al. (1993), Nguyen et al. (1997), and with halibut *(Hippoglossus hippoglossus)* by Grotmol et al. (1999) and Totland et al. (1999).

Since VER is usually detected in larvae and juveniles, even in hatcheries supplied with UV treated water, vertical transmission has been early suspected by several authors. It has been demonstrated that the virus may be present in ovaries and detected by ELISA (Arimoto et al., 1992), PCR or nested PCR (Mushiake et al., 1994; Nishizawa et al., 1996; Dalla Valle et al., 2000). The virus may be also present and detected by PCR in male gonads (Mushiake et al., 1994; Nishizawa et al., 1996). A few papers concerning detection of the etiological agent from unspecified gonads, using PCR (Nguyen et al., 1997; De Mas et al., 1998; Dalla Valle et al., 2000) and IFAT (Nguyen et al., 1997) have been published.

Surprisingly no virus isolation and culture has been reported from gonadal tissues, suggesting that infection is present at undetectable level with regard to the cell culture method. No report exists concerning viral isolation on cell culture from eggs. Nevertheless, fertilised eggs are often submitted to laboratory investigations and in a few occasions they have been found positive (Z. Peric, pers. comm.) Furthermore, two reports have been published on the use of ELISA to detect viral agent in fertilised eggs (Arimoto et al., 1992; Nishizawa et al., 1996).

Recently, the virus has been detected in the eggs and larvae originating from spawners experimentally (i.m.) infected with infectious material (Breuil et al., 2002). These results clearly show that infected parents may transmit infection to their offspring, but it is still to be proved whether the virus is located inside or outside the eggs. There are still some doubts on this subject and the final issue will depend on the efficacy of egg disinfection, in order to demonstrate if we may consider a real vertical transmission or an egg-associated transmission to occur. Preliminary results suggest that fertilised striped jack eggs may be effectively decontaminated by ozone or iodine treatment after experimental infection (Arimoto et al., 1996). These preliminary observations need to be confirmed by further experiments, particularly with naturally infected eggs and working with different species. Iodine compounds are routinely and successfully employed in aquaculture to disinfect salmonid eggs and to avoid or reduce the development of viral and bacterial infections in fry.

Some speculation exists on the possibility that vertical transmission could occur only in few species like shy drum *(Umbrina cirrosa)* and striped jack. No direct demonstration has been published so far, but the early development of infection, found to occur in 12-24 hour-old striped jack larvae (Mushiake et al., 1994) and 2 day-old shy drum larvae by IHC (G. Bovo, pers.obs.) strongly suggest that infection could initiate during embryogenesis. This hypothesis is supported by the positive reactions detected in ovaries of spawners by several authors.

Priority research needs regarding hazard identification

In order to avoid the spreading of diseases to new geographical areas it is recommended to avoid the distribution of contaminated eggs. Fortunately, in marine species juveniles represent the major commercial market for marine fish. This is mainly due to the critical first stage life, which needs special facilities and a complicate rearing system. For this reason hatcheries usually sell juveniles, which accept normal feed and are ready for on-growing farms. For the same reason, disinfection of eggs against VER is mainly a hatchery problem and of less relevance in commercial trade. Considering the actual situation, the priorities of future research should be the following:

1. Development of new susceptible cell-lines cultures. There is presently one cell-line which may be used for diagnostic purposes in VERV detection. It is absolutely necessary to prospect and maintain new cell-lines cultures to apply together with SSN-1.

2. Disinfection methods for eggs. Traditional methods using iodine should be investigated considering the different pH and salinity which may negatively affect the disinfection procedures in salt-water. Each fish species may show different sensitivity to iodine and it is therefore important to establish a safe dosage for each species to be treated.

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Infectious pancreatic necrosis

Disease description

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of young fish of salmonid species held under intensive rearing conditions. The causative agent, IPNV, is a bi-segmented double-stranded RNA virus belonging to the family Birnaviridae. The disease most characteristically occurs in rainbow trout *(Oncorhynchus mykiss),* brook trout *(Salvelinus fontinalis),* brown trout *(Salmo trutta),* Atlantic salmon *(Salmo salar),* and several Pacific salmon species *(Oncorhynchus spp.).* The disease has a wide geographical distribution, occurring in most, if not all, major salmonid-farming countries of North and South America, Europe and Asia. Susceptibility to clinical IPN generally decreases with age, with resistance to clinical disease in salmonid fish usually being reached at about 1500 degree-days, except for Atlantic salmon smolts, which can suffer from disease shortly after transfer from fresh water to seawater. Clinical signs include darkening pigmentation, distended abdomen and abnormal swimming behaviour.

Review of evidence for vertical transmission

a) Salmonid species

It is widely accepted amongst the international scientific community that vertical transmission of IPN is a typical characteristic of the disease in trout and possibly salmon. The published evidence for vertical transmission of IPNV via the fertilised egg of trout species is quite comprehensive and, in the main, conclusive but to some extent there is conflicting data, and as yet there is no clear consensus on the means by which the transmission occurs. In general terms the literature suggests that the probability of vertical transmission to the gametes is proportional to the virus levels in the gonads. A critical assessment of the published evidence for the different possible mechanisms of vertical transmission of IPN (and some other diseases) has been presented (in Japanese) by Egusa (1994).

The egg as a route of transmission of IPN in brook trout *(Salvelinus fontinalis)* was suspected following initial field observations on the disease in trout hatcheries in the USA by Snieszko et al. (1957) who had observed that it was not prevented by disinfection of the eggs at the eyed stage suggesting that the virus may be inside the egg. Then some years later, Wolf et al. (1963) directly demonstrated high levels of infectious virus in the ovarian fluids but to a much lesser extent in mascerates of washed eggs of some adult female brook trout suspected of being carriers and the source of outbreaks amongst fry. The authors observed that low levels of virus remained associated with washed ova and cautiously referred to this as 'egg-associated transmission' in the absence of evidence for the presence of the virus within the eggs. Later, Wolf et al. (1968) fertilised eggs from trout having IPNV in the ovarian fluid with sperm from males in which the virus was not detected, hatched the eggs in IPNV-free water and tested the hatched fry for presence of the virus. IPN occurred in a proportion of the fry and IPNV was isolated. This appeared to be confirmatory evidence for vertical transmission from carrier female trout but since the eggs were not disinfected, it cannot be concluded whether the virus was present within the egg or instead was persistently attached to the egg surface and infected the fry after hatching.

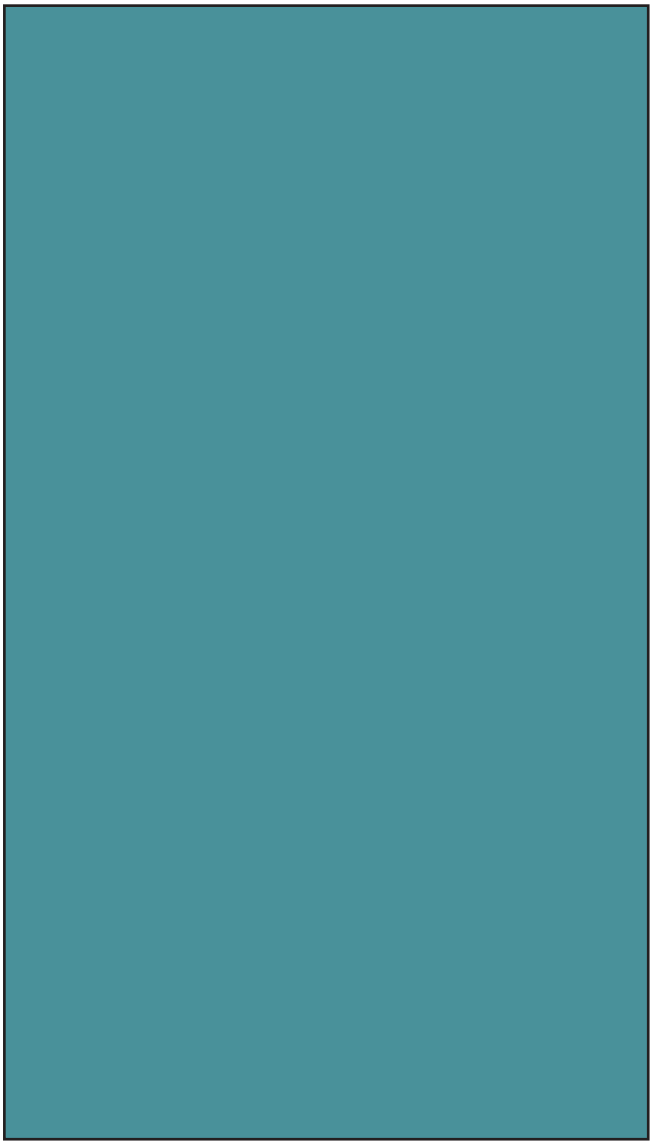
Tentative evidence for possible intra-ovum transmission was first presented by Bullock et al. (1976) who demonstrated that in carrier brook trout the virus was transmitted to the progeny (and resulted in mortalities) through eggs that had been disinfected at the eyed stage with an iodophor solution known to inactivate IPNV. It was concluded that IPNV had penetrated the egg or was present in a site within the shell that was inaccessible to the disinfectant. Subsequently, Fijan and Giorgetti (1978) claimed the first successful isolation of IPNV from rainbow trout *(Oncorhynchus mykiss)* eyed ova derived from a carrier brood stock population but, although the eggs had been washed, they had not been disinfected and it cannot not be ruled out that the virus was firmly adsorbed to the outer surface of the eggs rather than being present internally. Furthermore, Ahne and Negele (1985) later showed that eyed eggs of rainbow trout and Arctic char could be experimentally infected via IPNV-contaminated water and that the virus showed a strong affinity for the eggshell, so much so that infectivity of the virus persisted for more than 3 weeks, and even after hatching of the fry the eggshells were still infective. In contrast, rainbow trout eggs exposed to the virus before fertilisation or fertilised by sperm contaminated with the virus lost all infectivity after 60 minutes in flowing water leading the authors to suggest that the virus is not transported into the eggs through fertilisation with infected sperm but rather that contaminated eggshells may be the cause of 'egg-associated' transmission of IPN.

However, Dorson and Torchy (1985) using carrier rainbow trout which had been experimentally infected by intraperitoneal inoculation of IPNV, confirmed that the ovarian fluid contained infectious virus and then fertilised the eggs from those females with sperm from IPNV-free males. Even without disinfection of the eggs, the virus could not be detected in the developing eggs or in the hatched fry, indicating that the virus had not penetrated the eggs, or in some other way attached to an inaccessible site of the eggshell, to give vertical transmission. Bootland et al. (1991), using brook trout in which a carrier state had also been experimentally induced by intraperitoneal inoculation of IPNV, found that it was not possible to predict which parents would produce IPNV-infected progeny: failure to detect the virus in the reproductive products did not correlate with absence of vertical transmission nor did parents with virus detectable in the reproductive products always yield IPNV-infected progeny. The results provided no evidence for virus entering the egg whilst within the infected female parent but indirectly indicated that the source of the virus in the vertical transmission cases could be the sperm of carrier males.

The possible role of contaminated sperm in vertical transmission of IPNV was first speculated by Wolf et al. (1968) who detected the virus in the seminal fluid of a carrier male rainbow trout at spawning. Supporting indirect evidence was provided later by the detection of the virus in the seminal fluid of rainbow trout brood fish at a hatchery with a history of IPN (Ahne, 1983) and by the demonstration by Mulcahy and Pascho (1984) of the active adsorption of IPNV to rainbow trout sperm experimentally contaminated with the virus. The latter authors concluded that this supported the hypothesis that sperm from carrier males plays a role in vertical transmission of IPN. Further evidence for this was provided by Dorson and Torchy (1985) and Dorson et al. (1997) who achieved experimental egg transmission of IPN when spermatozoa were pre-incubated with relatively high concentrations of the virus and used to fertilise eggs from IPNV-free females. IPN developed in all groups of fry originating from eggs that had been fertilised with milt experimentally seeded with the virus and transmission was not prevented by disinfection of the fertilised eggs with iodophor after hardening. The authors concluded that this clearly demonstrated that IPNV was carried inside the egg by the spermatozoa. Additional evidence for adsorption of the virus to spermatozoa in the milt of naturally infected trout carriers was provided by use of flow cytometry analysis (Rodriguez et al, 1992).

From the many practical field observations and the types of experimental transmission work described above, there seems no doubt for trout species that IPNV can be vertically transmitted to the progeny of carrier broodfish. However, the evidence is not conclusive on whether this occurs only through true intra-ovum transmission via the embryo (from virus entering the egg either directly from the female or via the sperm of the male at fertilisation), or also through persistent IPNV contamination of egg surfaces not accessible to iodophor disinfection that are eaten by the fry at first feeding.

In contrast to the situation for trout, the evidence for vertical transmission of IPN in Atlantic salmon *(Salmo salar)* is sparse and inconclusive. It was shown many years ago that Atlantic salmon are susceptible to IPNV infection (MacKelvie and Artsob, 1969) but the relative rarity of reported outbreaks in hatcheries, and the results of experimental challenge studies (Hill, unpublished), show that the fry are much more resistant to the disease than are trout. In the 1980s, research in Norway indicated that clinical IPN in Atlantic salmon fry was relatively rare despite the detection of the virus in carrier fish (Melby et al.,1991) on most marine farm sites. However, in recent years there has been a steady increase in occurrence in freshwater sites in Norway and Scotland although by no means as much as that in marine farm sites (Krogsrud et al. 1989, Murray et al. 2003).



Indirect evidence for vertical transmission of IPN in Norwegian salmon hatcheries was presented by Krogsrud et al. (1989) even though the eggs and sperm had been taken from selected broodfish that tested negative for the virus. Smail and Munro (1989) examined the possibility of infecting the eggs of salmon via virus adsorbed to sperm at a rate of one virus particle per sperm. Virus was detectable inside the fertilised egg after pre-incubation of the contaminated sperm, which led the authors to conclude that sperm can cause virus entry into the egg. However, infectious virus could not be detected in the eggs an hour after water hardening. This transient detectable presence of infectious IPNV in eggs fertilised by contaminated sperm was confirmed in a second study (Smail and Munro, 1993) that also demonstrated that no virus was transmitted from experimentally infected carrier parents, or from carrier females on an IPNV infected salmon farm, to their eggs or the hatched progeny. The authors speculated that higher levels of infectious virus than those they detected in the ovarian fluid may be needed for vertical transmission to take place.

Whilst cases of IPN disease clearly do occur in fry in freshwater Atlantic salmon hatcheries, it is by no means conclusive that virus originates from broodfish in the marine sites via their eggs or sperm. Contamination of the hatchery environment with the virus by other means which results in horizontal transmission, cannot be ruled out in many cases. Further research is required to determine whether vertical transmission definitely does occur and under what conditions.

b) Non-salmonid species

There is very little published evidence, either indirect or direct, for vertical transmission of IPNV in other fish species than salmonids. In an early experimental study of the phenomenon of vertical transmission of IPNV in fish, Seeley et al. (1977) injected the virus into the peritoneal cavity of adult male and female zebra fish *(Brachydanio rerio)* and tested for the presence of the virus in fertilised eggs and progeny from various mating combinations. The results showed that transmission of the virus to the egg did occur via infected females but not via the sperm of infected males and the authors concluded that this confirms males do not play a role in transmitting IPNV to the egg. However, they did not speculate on the possible mechanism for the transmission via the female alone. Some years later, stripped bass *(Moxone saxatilis)* were shown to be naturally susceptible to IPNV (Schutz et al. 1984) and to become chronic carriers of the virus after experimental infection (Wechsler et al. 1986) but in a follow-up study to determine whether the virus could be spread vertically in this species, Wechsler et al. (1987) using experimentally-infected striped bass found no evidence for vertical transmission from carrier adults or via IPNV-contaminated eggs or sperm. Apart from these few published studies, there is no available evidence for or against vertical transmission of IPNV via eggs and/or sperm in freshwater non-salmonid species.

In marine fish, natural outbreaks of IPN disease have occurred in juvenile farmed turbot in France (Castric et al., 1987) and the virus has been isolated from moribund farmed turbot in Spain (Novoa et al., 1993). Experimental infection trials in the French study using intraperitoneal inoculation of high concentrations of the virus produced a 56% mortality in 3 month-old fish. In the Spanish study, experimental challenge by intraperitoneal inoculation of lower amounts of virus produced 100% mortality in small fish (2g). In Norway, the IPNV was associated with high mortalities in 2-3 month old turbot fry (Mortensen et al, 1990) but later experimental infection by the bath challenge method provided only inconclusive indication that turbot fry might be susceptible to infection by this route (Mortensen et al., 1993). In contrast, there is strong evidence that halibut *(Hippoglossus hippoglossus)* fry and juveniles are highly susceptible to IPN. High mortalities in newly metamorphosed halibut in early efforts with commercial production of this species were experienced in Norway and IPN was suspected to be a possible cause. The susceptibility of halibut to IPN was confirmed by Mortensen et al. (1990) who isolated the virus from 2-3 month old halibut fry suffering high mortality in a commercial farm in Norway. Experimental challenge studies confirmed the susceptibility of halibut fry to IPN (Ness et al., 1994; Biering et al., 1994) and subsequently a high mortality outbreak of the disease in farmed halibut juveniles was reported from Scotland (Rodger and Frerichs, 1997). Affected fish showed clinical and pathological signs consistent with those of IPN in salmonids. However, despite the importance of IPN as a problem for farming of turbot or halibut, no evidence has yet been presented to indicate that vertical transmission may occur in these species.

Priority research needs regarding hazard identification

1. Further research is needed to resolve the uncertainty over whether vertical transmission in trout occurs through true intra-ovum transmission via the embryo as a result of virus entering the egg. It needs to be clarifyed of this transmission occurs either directly from the female IPNV carrier or via the sperm of carrier males at fertilisation, or if it can occur by either route.

2. If it is confirmed unequivocally that vertical transmission does take place through contaminated sperm, studies are needed to determine the lower threshold concentration of the IPNV in the seminal fluid for transmission to take place.

3. More research is needed on Atlantic salmon, along the lines of that conducted for trout, to clarify whether egg-associated or true vertical transmission of IPNV occurs for this species and under what conditions. This is an important issue, particularly in view of

the probable development of commercial trade in sperm of salmon with improved genetic characteristics for aquaculture.

4. More sensitive methods need to be developed for detection of carrier salmonid broodfish and for rapid quantification of infectious virus in the sexual products to determine which individual fish may safely be used to produce fertilised eggs with no risk of IPN disease developing in the progeny.

5. Studies are needed to determine whether vertical transmission occurs in any of the major farmed non-salmonid marine species known to be susceptible to IPN and for which a trade in eggs or sperm could feasibly develop.

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Bacterial kidney disease *(Renibacterium salmoninarum)*

Disease description

Bacterial kidney disease (BKD) is a typically chronic infective disease caused by *Renibacterium salmoninarum,* a coryneform Gram-positive bacterium that is the sole species belonging to the genus *Renibacterium* and that has been reported to occur in North America, Japan, Western Europe and Chile. It is of economic importance to salmonid husbandry, especially with regard to Pacific salmon *(Oncorhynchus* spp.), because of its widespread distribution both in freshwater and saline environments, its chronicity, which does not allow the disease to be suspected before late clinical or debilitating manifestations, and the inefficacy of the main therapeutic compounds used for treating infected fish.

Review of evidence for vertical transmission

Renibacterium salmoninarum is presently the best-documented fish bacterium as regards the occurrence of vertical transmission of infection through sexual products. The disease has been studied since a long time, and according to Fryer and Sanders (1981), Allison, as early as 1958, was the first author to suspect that the infection was transmitted from contaminated to disease-free fish farms after introduction of salmonid eggs. This did not provide any indication, of course, about the location of the bacteria on or within the eggs. Whereas veterinarians commonly considered that vertical transmission *stricto sensu* may sometimes result from egg-surface contamination, and so may be prevented by convenient disinfection procedures, epidemiologists are prone to restrict the concept to the internalisation of pathogens in egg annexes (*in ovo* contamination), that renders such a procedure much less effective. Hence, only the last meaning will be taken in consideration in the following discussion.

The first observations to support the possibility of *in ovo* contamination with *R. salmoninarum* came from Bullock et al. (1978), who could demonstrate the persistence of BKD infection in chinook salmon and rainbow trout issued from the same broodstocks, after iodine-disinfected eggs had been transferred to 2 different locations from West Virginia and Washington. Evelyn et al. (1984a, 1984b, 1986) brought a series of experimental confirmations, establishing that iodine-disinfection performed during or after water-hardening of eggs was no more complete, and providing microscopical pictures to support a possible presence of bacterial cells in the vitellus. The intra-ovum penetration of the agent was deemed to occur passively from the surrounding infected coelomic fluid, seemingly via the micropyle, while an active role of male sperm during fecundation could not be substantiated. Bruno and Munro (1986) reached the same conclusions after microscopy studies that seemed to unveil the presence of bacteria in the ovary and even inside oogonia of rainbow trout, albeit in very early stages of sexual maturation. Later, Brown et al. (1990) afforded a fine demonstration of experimental egg infection, using a microinjection technique which resulted in the development of BKD infected progeny.

A common agreement in all these experiments is the significance of the coelomic fluid contamination for egg-infection process. Strong correlation was generally noticed between the bacterial load of coelomic fluid and the resulting rate of egg contamination (Evelyn et al. 1984a, Lee and Evelyn 1989). Contamination, however, may eventually be considered to represent a rare event (Evelyn et al.1986), and conversely it may happen to occur unexpectedly from apparently healthy individual females (Lee et al. 1989).

The mechanisms involved in *R. salmoninarum* penetration and survival in salmonid eggs presently remain highly hypothetic. Innate defence mechanisms are still poorly documented in fish eggs, although molecules likely to express antimicrobial properties have occasionnally been described. This is the case for a lectin isolated from chinook salmon ova (Voss et al. 1978), and for lysozyme, which was demonstrated to be present in coho salmon eggs at sufficient concentrations to inhibit several fish associated bacteria development (Yousif et al. 1994). Interestingly, *R. salmoninarum* was resistant to this lysozyme activity, even at low temperatures, and this property is important to support the ability of the bacterium to be transmitted vertically.

Considering the bulk of experimental work conducted on BKD, vertical transmission demonstration appears convincing, even though additional details would be welcome for a better understanding of the involved mechanisms. Two series of additional arguments are worth being briefly mentioned to complete this overview. On one hand, an impressive set of detection methods have been developed for testing broodstock fish health status, and in practical applica-

tion, many of the subsequent studies have brought additional and confirmatory information for the effective presence of *R. salmoninarum* inside salmonid fish eggs. On the other hand, and perhaps more significantly, control programmes based on the treatment of maturing fish with antibiotics such as erythromycin or on routine screening and selection of uninfected or moderatly infected breeders before artificial fecundation have been widely practised. Indeed they resulted in noticeable decrease of the disease prevalence in formerly heavily affected salmonid populations. As these particular aspects are intended to be specifically detailed in further work-packages, they will not be considered here. They represent, however, indirect confirmations and additional proofs for the significance of vertical transmission in BKD epidemiology.

Priority research needs regarding hazard identification

It is clear from the above summary that *R. salmoninarum* is one of the fish pathogenic agents to which most attention has been paid for establishing the reality of intra ovum infection and vertical transmission. Experimental investigations have been thoroughly conducted, and there is no doubt that clues in favour of the transmission of BKD from parents to progeny are now quite convincing.

Future developments should now address:

1. The exploration of basal mechanisms commanding the interrelationships between the bacteria and the protection mechanisms that may exist in eggs, as well innate properties as possibly acquired protection from maternal origin.

2. Regarding hazard identification and risk analysis approaches, a critical point could be the collection of more quantitative data, which presently remain scarce or limited to adult fish populations. This means that epidemiological studies should be encouraged. They may be mainly descriptive at first, aiming at collecting enough information to provide eventually useful indicators about the rates of infection and their most likely evolution in different environmental and biological circumstances. This seems to be an important prerequisite to sustain future probabilistic analyses.

3. Progress may also be expected from a better perception of the biological interactions between *R. salmoninarum* and other egg-associated bacteria. Microbial flora has been shown to vary according to the stage of development of eggs, and *R. salmoninarum* does not seem to be the only organism to express a faculty of penetration inside the eggs (Bell et al., 1971; Barker et al., 1989, Sauter et al., 1987). Perhaps it would be possible to take profit of microbial competition and strengthen the set of control measures presently available for BKD prevention?

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Infections with Gram-negative bacteria

Flavobacterium psychrophilum infections

Disease description

Flavobacterium psychrophilum, the agent of the rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (when affecting larger fish), has been known in the USA since 1946. It was detected in Europe in 1984-1985 and is now considered to be widespread throughout Europe. RTFS occurs in fresh water at temperatures of 4-12^{0}C, losses decreasing at higher temperatures. Fry affected by RTFS show lethargy and increased pigmentation, loss of balance, ascites and abdominal swelling. Vertebral deformities are frequently observed in rainbow trout surviving early *F. psychrophilum* infection. Larger fish most often exhibit subcutaneous lesions which tend to become extensive and develop into deep ulcerations in dorsal musculature ("saddleback disease") or tail fin necrosis ("peduncle disease").

Review of evidence for vertical transmission

Vertical transmission of *Flavobacterium psychrophilum* is still a matter of controversy. Although most of the authors of published papers are fully convinced that it regularly occurs in field situation, experimental attempts to demonstrate this property have not yet produced definitive evidence. Before addressing to the reasons for which it is so difficult to solve the question, compared to the relatively well documented case of *R. salmoninarum,* we shall try to give an account of the different data presently available.

A series of observations come at first from epidemiology. The rainbow trout fry syndrome (RTFS) appeared very suddenly in Europe in 1984, and it immediately became a major source of concern. It is striking to note that in less than 3 years, independent descriptions of this so far unfrequent form of the disease were recorded from Germany, France, Italy and Denmark (Lorenzen et al. 1997). This does not provide useful indications on a possible role of sexual products, but it suggests a sudden occurence of a new pathogen rather than the late discovery of a previously unsuspected agent. It is of interest to note that the earliest documented cases from Japan approximately occurred in the same period (1987), after importation of coho salmon eggs from the United States (Wakabayashi et al. 1991). Wakabayashi et al. (1994) established that the isolates associated to coho salmon infection were of the same serotype as those already reported from American stocks, whereas a different serotype was identified in ayu *(Plecoglossus altivelis).* Beyond this special example, as international trade exchange of live salmonids is generally less developed than direct consignement of eggs, the assumption of an egg-associated propagation of the infection was worth being considered.

Borg (1960) was apparently the first author to suspect a possible transfer of coldwater disease from a location to another through introduction of fertilised eggs. In his Ph.D. thesis, Holt (1987) accounted for the frequent isolation of *F. psychrophilum* in the coelomic fluid of infected females (about 38 % of the examined samples) and defended the hypothesis that this bacterium could be transmitted vertically. Further observations came from Symula et al (1990), who studied the mortality associated to a form of blue sac disease in the Lake Ontario *Salvelinus namaycush* populations. They reported both a "female-effect" and the apparent presence of *F. psychrophilum* "inside" the eggs. They could also detect the bacterium in the milt of certain males, together with other bacteria, but although they clearly rendered bacteria responsible for the observed mortality they did not draw premature conclusions about a possible in ovo contamination of the progeny with *F. psychrophilum.* Cipriano et al. (1995) were more affirmative and established a direct link between the mortality of eyed Atlantic salmon eggs and the isolation of *F. psychrophilum.*

Experimental confirmation of an association of *F. psychrophilum* to rainbow trout eggs and to recently hatched fry was achieved by Lorenzen (1994), who considered, however, the contamination to occur only on the egg surface. Rangdale et al. (1996, 1997) completed this evidence through a series of studies conducted in English RTFS-infected sites. The bacterium was recovered from ovarian fluids, from eggs, but not from sperm. Experimental infection of eggs also resulted in the development of RTFS in fry. According to these authors, vertical transmission appeared very likely, although Cytophaga-like and *Pseudomonas* species were also commonly associated to coelomic fluid, as already noticed by Barker et al. (1989).

All these works, completed with the demonstration of strong adhesive properties of *F. psychrophilum* to different supports including

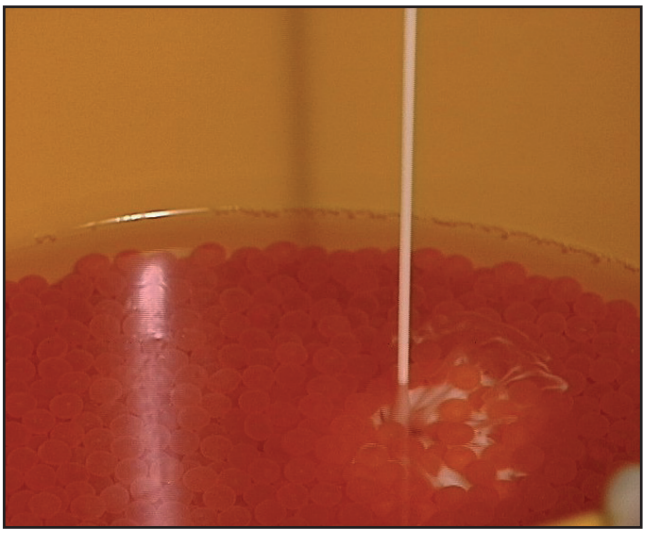
egg surface (Vatsos et al. 2001a), had brought some presumption about the contamination of sexual products and the likely transmission of the bacterium from parents to progeny in the absence of disinfection. Nevertheless, convincing evidence for a true vertical transmission was still lacking, when two important series of investigations were respectively reported from Japan and North America. Using a nested PCR in comparison to indirect immunofluorescence and to bacterial culture, Izumi and Wakabayashi (1997) were able to detect *F. psychrophilum* in 5 out of 7 consignements of coho salmon eggs imported from the USA. Moreover, iodine disinfection of the eggs did not prevent the detection of the bacterium (Kumagai & Takahashi 1997). Experimental exposure of eggs to the bacterium (Kumagai et al. 2000) resulted in similar issues, showing that eggs could become infected only when exposed before water-hardening had occurred, and that the bacterium, which was also demonstrated to be resistant to lysozyme activity, could then be detected only in the egg content, never in the chorion.

Brown et al. (1997) featured a very complete experimental schedule to compare rates of infection in salmon eggs before and after water-hardening, after applying different procedures for control: absence of treatment, injection of females with erythromycine or tetracycline prior to fecundation, iodine-disinfection of eggs. The subsequent results strongly supported the survival of the bacterium inside the eggs and the colonisation of the alevins, namely when contamination occured by contact with infected coelomic fluid before water-hardening. The sterilization with iodine was shown to require at least 1 h to be complete. Adult female treatment did not provide consistent results, and an unusual resistance to lysozyme was also noticed. Although unexpected water-borne reinfection occurred, the source of which remained unexplained, these results were in good accordance with those of the japanese authors. Together, they afford our strongest evidences to the probability of *in ovo* transmission of *F. psychrophilum.*

No special observation was added in a later survey conducted in Atlantic salmon by Ekman et al. (1999). Gamete association was confirmed, without indication of internal penetration of the bacterium. The afore-mentioned conclusions were more seriously questionned, however, by the results of Danish workers (Madsen et al. 1999; Madsen & Dalsgaard 2001; Dalsgaard & Madsen 2002) who used nested PCR in repeated attempts but failed to detect the presence of the bacterium inside the eggs, even after experimental exposure. They concluded that surface contamination was most probably involved in RTFS transmission.

This apparent difficulty to reach an agreement about the ability of *F. psychrophilum* to penetrate inside fish eggs and infect salmonid progeny may appear somewhat surprising. Certainly, the bacterium is known for its special temperature and nutritional requirements and its culture calls for special media, but so is the case of *R. salmoninarum,* too, which never aroused so controversial interpretations. Several characteristics may partially explain this situation.

It is clear that direct isolation should represent the best way of asserting the existence and survival of the bacterium inside the eggs. Culture of *F. psychrophilum,* however, presents a particular inconstancy. Certain strains properties or certain environmental conditions are likely to interfere with its growth, and the ability to produce viable but non-cultivable forms (VNC) has been strongly suspected (Michel et al. 2000, Vatsos et al. 2001b). In such conditions, microscopy or indirect detection methods should appear more convenient for intra-ovum detection. As yet, curiously, almost all approaches have been conducted using PCR, which is considered the most sensitive technique but also presents some drawbacks. Amplification may be hampered by the presence of inhibitory components that are strongly suspected to occur in fish egg content, and the risk of contamination with surrounding materials, during the steps of egg content extraction, cannot be completely disregarded. As long as complementary tests using independent techniques will not afford confirmation of the published observations, it will be difficult to settle apparent inconsistencies and to reach definitive conclusions.



Iodine disinfection, which has been commonly used in the reported experimental investigations to make sure that external microorganisms would not interfere with the analysis of egg contents, is also a matter of controversy. Brown et al. (1997) have noted that the doses and exposure time had to be increased to ensure complete inactivation of the *F. psychrophilum* bacteria. Currently used disinfection procedures are far from respecting such requirements in practice. If confirmed, both the difficulty in demonstrating the reality of intra-ovum transmission of *F. psychrophilum* and the dramatic spreading of the infection at world scale between 1984 and 1992 could be explained.

Priority research needs regarding hazard identification

Two main domains of knowledges could clearly benefit from further investigations to better understand the mechanisms of vertical transmission of *F. psychrophilum* and substantiate future risk assessment approaches.

1. The first one is related to the still poorly known properties of the bacterium. Cultural requirements have been actively investigated in the recent past years, and the improvement of media formulations has indeed resulted in a better control of the bacterial production in artificial media. The possible existence of VNC calls, however, for additional studies. It would be namely critical to analyse the behaviour of the bacteria and the structure of the resulting population, as well in fish and biological tissues as in natural habitat. Survival of *F. psychrophilum* in the environment, is still poorly documentet and it may be assumed that its strong adhesive properties, together with the classical production of exo-polysaccharides by all members of the gliding bacteria phenon, could result in the special ability to colonize optimal microhabitats and perhaps participate to biofilms formation on convenient structures, including the egg surface.

2. The second challenge is to develop convenient techniques to solve definitively the question of the bacterium survival in fish eggs. PCR does not seem to be sufficient for such a purpose, and it would perhaps be profitable to rely more upon microscopical techniques. Another promising method could be to develop RT-PCR and try to detect the expression of mRNAs rather than DNA, in order to assess that the detected bacteria are really alive. This, of course, would suppose a thorough step of adaptation and optimisation of the techniques.

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Infections with rickettsia-like organisms (RLOs)

Piscirickettsiosis *(Piscirickettsia salmonis)*

Disease description

Piscirickettsiosis, also called salmonid rickettsial syndrome (SRS) is a septicaemic infection of salmonid fish caused by a Gram-negative, fastidious, intracellular bacterial pathogen named *Piscirickettsia salmonis*. The disease was first described in coho salmon *(Oncorhynchus kisutch)* and is known to affect other Pacific salmon species including rainbow trout *(Oncorhynchus mykiss),* and Atlantic salmon *(Salmo salar).* Most frequently, the disease occurs several weeks after sea transfer and recurrent clinical outbreaks causing very high cumulative mortality (30-90%) have been reported from Chile where it was first described. It has also been reported from Atlantic and Pacific Canada; from Ireland, Scotland and Norway. Clinical disease has also been reported in fish approaching sexual maturation (Fryer and Lannan 1996).

Review of evidence for vertical transmission

The first scientific report suggesting that *P. salmonis* may be transmitted vertically was presented orally by Bustos et al. (1994). The authors reported severe *P. salmonis* infection in nearly 100% of fullsib smolts derived from a female coho salmon that had tested positive for *P. salmonis* by IFAT at the time of stripping. A parallel group of siblings from another, IFAT-negative, female yielded a *P. salmonis* prevalence of 26.7% but only low-intensity infection. Both females had been fertilised with sperm from males testing negative by IFAT. Due to the fact that the eggs from each parental cross had been incubated in UV sterilised water and the offspring had been held completely separate from fertilisation through smoltification, the authors concluded that vertical transmission had occurred and it was suggested that control measures against vertical transmission should be implemented.

The first reports of piscirickettsiosis outbreaks in fresh water was by Bravo (1994) and Gaggero et al. (1995) but the underlying epidemiological data did not allow for a firm conclusion as to which was the likely route of infection.

The presence of *P. salmonis* organisms inside fertilised ova from experimentally infected rainbow trout was reported by Larenas et al. (1996). In four fullsib groups derived from crossing negative or positive males or females, the prevalence of fertilised eggs testing positive for *P. salmonis* by IFAT when sampled 18 days after fertilisation was 6.7-10% (one or both parents positive) and 0% (both parents negative), respectively. Observational data from naturally infected coho salmon have since then been presented showing that vertical transmission of *P. salmonis* may occur under normal farming conditions (Larenas et al. 1999).

Despite these reports, vertical transmission of piscirickettsiosis was until to date considered obscure within the international scientific community (anonymus, 2000). In a recent review of the disease (Mauel & Miller 2002) the low incidence of disease observed in freshwater reared fry and fingerlings is seen contradictory to a significant role for vertical transmission, a view shared also by Lannan et al (1999). This is likely due to the fact that the mentioned findings – as yet – were not published in an international peer review journal.

This is currently being rectified as a manuscript presenting the outcome of rather extensive experimental studies done in Chile has been made available to this project group. Therein is being confirmed that *P. salmonis* may be found in the ovarian and seminal fluids of *P. salmonis*-positive rainbow trout parents, and that eggs fertilised with sperm from positive males yielded infected offspring despite normal disinfection procedures. The same authors also reported vertical transfer following in vitro infection of gametes from *P. salmonis*-negative parents during fertilisation (Larenas et al. 2002). The results were supplemented through electron microscopy studies showing how piscirickettsial organisms attach to and are being embedded into the surface of the egg (Larenas et al., 2003) indicating that penetration of *P. salmonis* through the chorion occurs via active transpor and by a very rapidly acting mechanism.

Based upon this evidence, vertical transmission of *Piscirickettsia salmonis* – including the vertical transfer of viable infectious organisms inside the fertilised salmonid egg – so-called "true vertical transmission" should now be considered scientifically established.

Priority research needs regarding hazard identification

In order to further confirm the vertical transfer of *P. salmonis* inside the fertilised eggs of salmonids and to elucidate the underlying biological mechanisms, the following subjects should be prioritised in future research:

1. Studies of *in vitro* attachment of *P. salmonis* to the sperm of various salmonid species.

2. Transmission studies in experimentally or naturally infected coho salmon and Atlantic salmon breeders, including *in vitro* infection of gametes from these species.

3. The repetition of studies showing the presence and density of viable *P. salmonis* organisms in seminal and ovarian fluid, and inside fertilised eggs from naturally or experimentally infected salmonids. Studies in the Atlantic salmon are particularly welcome.

4. Confirmation of the attachment and entrance of *P. salmonis* into the salmonid egg (mode, speed and mechanisms of penetration).

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Summary and conclusions

The project group has, from the OIE list of notifiable diseases of finfish, and the EU finfish diseases on list I and II, selected ten infections for in-depth scrutiny (attachment 1).

Focus of this report has been to establish the evidence – or lack of such – that vertical transmission of the selected diseases does actually occur and in particular if transmission may occur inside the fertilised egg ("true vertical transmission") or only as contamination of the egg surface ("egg-associated transmission"). The results suggest that evidence for true vertical transmission has been presented for BKD, IPN and Piscirickettsiosis, whereas experimental and field studies suggest that vertical transmission of VHS, IHN and ISA may be effectively prevented by common egg disinfection procedures. For several of the listed diseases, there is a lack of published information.

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Attachment 1:
List of fish diseases for review

Infectious haematopoietic necrosis (IHN)
Viral haemorrhagic septicaemia (VHS)
Spring viraemia of carp (SVC)
Epizootic haematopoietic necrosis (EHN)
Red sea bream iridoviral disease
White sturgeon iridoviral disease
Infectious salmon anaemia (ISA)
Viral encephalopathy and retinopathy/Viral nervous necrosis (VER/VNN)
Infectious pancreatic necrosis (IPN)
Bacterial kidney disease (BKD)
Flavobacterium psychrophilum infections
Piscirickettsiosis