



Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*

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ABSTRACT: Cardiomyopathy syndrome (CMS) is a disease of unknown aetiology, having significant economic impact as it primarily affects large, farmed Atlantic salmon *Salmo salar* L. in seawater, close to harvest. In the present study, we have demonstrated that CMS is a transmissible disease under experimental conditions. Histopathological lesions consistent with CMS were induced in Atlantic salmon post-smolts after injection of tissue homogenate from farmed fish diagnosed with CMS. Six weeks post-injection (p.i.), experimental fish started developing focal to multi-focal lesions in the atrial endo- and myocardium, with subsequent progression to the ventricle. This proceeded into severe endocarditis and subsequent myocarditis with mononuclear cell infiltration of the atrium and, to a lesser degree, the spongy layer of the ventricle. These lesions were consistent with histopathological findings in field outbreaks of CMS. From Week 33 p.i., lesions also appeared in the compact myocardium, with focal epicarditis adjacent to focal myocardial lesions. In conclusion, these results indicate that CMS has an infectious aetiology and should be treated as a potentially contagious disease.

KEY WORDS: Atlantic salmon · *Salmo salar* · Cardiomyopathy syndrome · CMS · Experimental transmission · Myocarditis · Pathology · Cardiomyopathy · Transmission

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INTRODUCTION

Cardiomyopathy syndrome (CMS) is a severe cardiac disease of unknown aetiology in farmed Atlantic salmon *Salmo salar*. It was first diagnosed in 1985 in Norway, and later in Scotland and the Faeroe Islands (Amin & Trasti 1988, Bruno & Poppe 1996, Rodger & Turnbull 2000). Suspicious cases have also been reported from Canada (Brocklebank & Raverty 2002). Outbreaks of CMS in farmed fish occur along most of the Norwegian coast, with the highest number of affected sites in mid-Norway (Kongtorp et al. 2006a). Most CMS cases are diagnosed during the late seawater phase; typically from 14 to 18 mo after sea transfer. Mortality may be moderately elevated over a long period, or suddenly high without prior symptoms (Ferguson et al. 1990, Brun et al. 2003). As CMS generally affects large fish, the economic losses may be substantial, even though the cumulative mortality may be

low or moderate (Brun et al. 2003, Østvik & Kjerstad 2003).

Diagnosis of CMS is based on clinical findings, autopsy and histopathology. At autopsy, fish with CMS typically show skin haemorrhaging, raised scales and exophthalmia. Ascites and fibrinous casts on the liver surface are also common. The atrium and sinus venosus are usually enlarged, sometimes ruptured, and blood or blood clots often fill the pericardial cavity (Bruno & Poppe 1996). Histopathologically, CMS is characterised by inflammation and necrosis of endocardium and spongy myocardium in the atrium and ventricle. Cellular infiltrates consist mainly of mononuclear cells, most probably lymphocytes and macrophages. The compact myocardium is usually not affected, but epicardial cell infiltrates may extend into the compact layer along branches of the coronary vessel (Ferguson et al. 1990). Lesions may progress to such a state that the wall of the atrium or sinus venosus

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weakens or ruptures, with resultant haemopericardium and death. Previous studies indicate that CMS is a chronic disease developing over a period of several months prior to the terminal clinical phase (Ferguson et al. 1990, Kongtorp et al. 2006a).

Several hypotheses on the cause of CMS have been put forward, including environmental, immunological and microbiological factors (Kongtorp et al. 2005). Most of these hypotheses have not been studied further. Cardiac lesions in CMS may resemble pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI), although the diseases are histopathologically distinguishable in typical cases (Kongtorp et al. 2004b, 2006b, McLoughlin & Graham 2007). PD is caused by salmonid alphavirus (McLoughlin et al. 1996, McLoughlin & Graham 2007). HSMI is experimentally transmissible and thought to be of viral aetiology (Kongtorp et al. 2004a). Both diseases cause severe myocardial inflammation and necrosis. Due to similarities with PD and HSMI, and the widespread occurrence of CMS, a viral aetiology has been suggested, although attempts to isolate virus from tissue sampled from fish with CMS have not yet been successful (Kongtorp et al. 2005). The aim of the present study was therefore to investigate the transmissible nature of CMS under experimental conditions.

MATERIALS AND METHODS

Experimental fish. A total of 496 healthy Atlantic salmon smolts *Salmo salar* of a wild strain were used as experimental fish. The fish had been hatched and grown in a fresh water cultivation facility (Hellefoss) in eastern Norway, geographically isolated from the Norwegian population of farmed fish. The fish were not vaccinated, and the cultivation facility had no history of disease. The experiment was performed at the Norwegian Institute of Water Research (NIVA), Solbergstrand, Akershus. Both the cultivation and research facilities are situated in an area with no commercial fish farms, physically and geographically well separated from the endemic area of CMS in Norway. The research facility was approved for challenge experiments with unknown, suspected infectious agents by the Norwegian Food Safety Authority. All effluent water was filtrated (filter pores $\geq 300 \mu\text{m}$) before being treated with hypochlorite, resulting in a total concentration of chlorine in effluent water of at least 35 mg l^{-1} 30 min after treatment.

The fish were transported to the research facility (approximately 3 h transport time) in a water tank with oxygenation 10 wk before commencement of the experiment. There was no transport-related mortality. The fish had not been exposed to seawater, but most

fish had lost their parr markings and had a silvery appearance. The average length and weight were 17 cm and 35 g, respectively, a fish size which fit well with the capacity at the facility.

Husbandry. At the research station, the fish were housed indoors in a fibreglass tank. Osmoregulatory capacity was tested after 2 wk of acclimatisation by exposing 7 fish to full salinity seawater for 24 h. After sedation in chlorobutanol (300 mg l^{-1}), blood was sampled from the caudal vein of these fish, the fish were euthanized by decapitation and the chloride concentration was measured (Central Laboratory, Norwegian School of Veterinary Science) (Eisenman 1967, Tietz 1995). As the results indicated smoltification, the experimental fish were transferred to seawater 7 d later. After another 5 d, the fish were transferred to the experimental tanks.

During the experiment, the fish were kept in cylindrical fibreglass tanks with a conical bottom and central drainage, containing approximately 1.35 m^3 of water. Water flow was 300 l h^{-1} , providing a complete water exchange every 4.5 h. Seawater was pumped from a depth of 60 m. Mean temperature was 8.5°C (range: 7.1 to 10.2°C) and mean salinity 33.8‰ (range: 32.7 to 34.4‰). The fish were fed a commercial pelleted feed with automatic feeders, at a feeding ratio of approximately 1% body weight d^{-1} . A photoperiod regime of 10:14 h light:dark was used. Mortalities were registered, collected and stored at -18°C , and fish showing aberrant behaviour were killed by a blow to the head and decapitation before collection and similar storage.

Preparation of inoculates. Tissue homogenate: Samples of cardiac and kidney tissue homogenates from 6 Atlantic salmon collected during 2 field outbreaks of CMS were pooled and used for inoculation. Four of these fish were found dead in the cages and displayed severe inflammation and necrosis of endo- and myocardium in spongy tissue of the atrium and ventricle on histopathological examination. The other 2 fish showed normal swimming behaviour and were caught live from the same cage as 2 of the dead fish. These fish had only mild inflammation in cardiac tissue. Tissue samples were diluted 1:10 in Leibowitz L-15 cell culture media, homogenised and centrifuged at $2500\times g$ for 7 min. The supernatant was further diluted 1:2 in L-15 supplemented with gentamycin (final concentration: $50 \mu\text{g ml}^{-1}$) before inoculation.

Negative control inoculate: Leibowitz L-15 cell culture media, supplemented with gentamycin ($50 \mu\text{g ml}^{-1}$) was used as the negative control inoculate.

Challenge. The study was initiated after a 2 wk acclimatisation period in the experimental tanks. Experimental fish were randomly allocated to 4 groups of

approximately 100 fish (range: 92 to 102), each group in a separate tank. Injection of inoculates was performed after sedation in chlorobutanol (300 mg l⁻¹). Duplicate groups of challenged fish (denoted Challenges 1 and 2) were injected intraperitoneally (i.p.) with 0.2 ml supernatant from tissue homogenate. Similarly, duplicate groups of negative control fish (Controls 1 and 2) were injected i.p. with 0.2 ml of negative control inoculate.

Sampling. Samples for histology, real-time reverse transcription-polymerase chain reaction (RRT-PCR) and microbiology were collected from 5 experimental fish before commencement of the study. Post-challenge, sampling was performed every 3 wk for a period of 42 wk, resulting in a total of 14 samplings. At each sampling, 5 experimental fish were collected from each group. Sampled fish were anaesthetised in chlorine butanol (300 mg l⁻¹) and killed by decapitation. Samples for RRT-PCR and cell culture were stored at -80°C until results from the histopathological examination had been finalised.

As the number of fish in Control Group 1 was greatly reduced by an outbreak of infectious pancreatic necrosis (IPN), sampling from this group was not performed from Week 15 to Week 33. The last fish in this group were sampled 36 wk post injection (p.i.).

Histopathology. Tissue samples from gill, pseudo-branch, heart, liver, pyloric caeca with pancreas, mid-kidney, spleen and skeletal muscle were fixed in 10% neutral phosphate-buffered formalin and prepared by paraffin wax embedding and standard histological techniques (Bancroft & Stevens 1990). Sections were stained with haematoxylin and eosin (H&E).

Sections of cardiac tissue were classified histologically based on the presence of mononuclear endo- and myocarditis, degeneration and necrosis in the spongy layer of the ventricle and atrium (Ferguson et al. 1990). The atrium, epicardium, compact and spongy layers of the ventricle and the endocardium of both cardiac compartments were examined and evaluated. The findings were graded from 0 to 4 according to the criteria in Table 1 (see also illustrations in Fig. 1).

Microbiology. Bacteriology: Swabs from mid-kidney of 2 fish in each sampled group were cultivated on blood agar plates with and without 2% NaCl at 15 and 22°C, respectively, for at least 6 d. Similar cultivation was also performed on samples from both mid-kidney and skin lesions from fish with skin ulcerations. Standard procedures for identification of isolated bacteria were performed.

RRT-PCR: After completion of the experiment, 76 fish were examined for piscine nodavirus, salmonid alphavirus 3 (SAV3) and infectious pancreatic necrosis virus (IPNV) by RRT-PCR. The fish examined were those used for inoculate preparation, all fish from the

Table 1. *Salmo salar*. Histological classification of lesions in endo-, epi- and/or myocardium

Score	Description
0	No pathological findings, or slightly increased number of leukocytes
1	One or a few focal lesions, increased number of leukocytes
2	Several distinct lesions and small to moderate increase in number of leukocytes
3	Multifocal to confluent lesions and moderate to severe increase in number of leukocytes
4	Severe confluent lesions comprising >75% of the tissue and massive leukocyte infiltration

pre-challenge sampling, a selection of mortalities, all fish from 3 scheduled samplings and some fish with severe heart lesions from scheduled samplings (see Table 2). Total nucleic acids were extracted from heart and kidney tissue using the NucliSens® easyMAG™ on-board protocol (bioMerieux) according to the manufacturer's instructions. The nucleic acid concentrations were determined using a Nanodrop ND-1000 (NanoDrop Technologies). Detection of nodavirus by RRT-PCR was performed according to Grove et al. (2006), but without quantification. RRT-PCR for detection of SAV (Jansen et al. 2007) and for detection of IPNV (Ørpetveit et al. 2007) was also performed. All RRT-PCR reactions were performed on a Stratagene Mx3005P.

Cell culture: Post-experiment, heart and kidney tissue from 6 challenged fish sampled at 12, 18, 24 and 27 wk p.i., all with moderate to severe (Grade 3 or 4) CMS-like histopathological atrial lesions and at least Grade 2 lesions of the spongy myocardium of the ventricle, were examined in cell cultures according to routine procedures at the National Veterinary Institute, Oslo, Norway. The tissue samples were homogenised in cell culture medium (w/v 10%), and the homogenates were cleared by low-speed centrifugation. As IPNV is ubiquitous in Norwegian salmon farming (Melby et al. 1991, Jarp et al. 1995, 1996), the homogenates were treated with a mix of polyclonal neutralising antibodies against IPNV serotype Sp and serotype Ab. The homogenates were then inoculated onto cell cultures from bluegill fry fibroblast (BF)-2 cells (Wolf & Quimby 1966), epithelioma papulosum cyprini (EPC) (Fijan et al. 1983), rainbow trout gonad (RTG)-2 (Wolf & Quimby 1962), chinook salmon embryo (CHSE)-214 (Lannan et al. 1984) and Atlantic salmon head kidney (ASK) (Devold et al. 2000). Inoculated cells were incubated for 1 wk at both 15 and 20°C in parallel and were regularly investigated with an inverted microscope for the occurrence of a cytopathic effect (CPE). After 1 wk, the supernatants were pas-

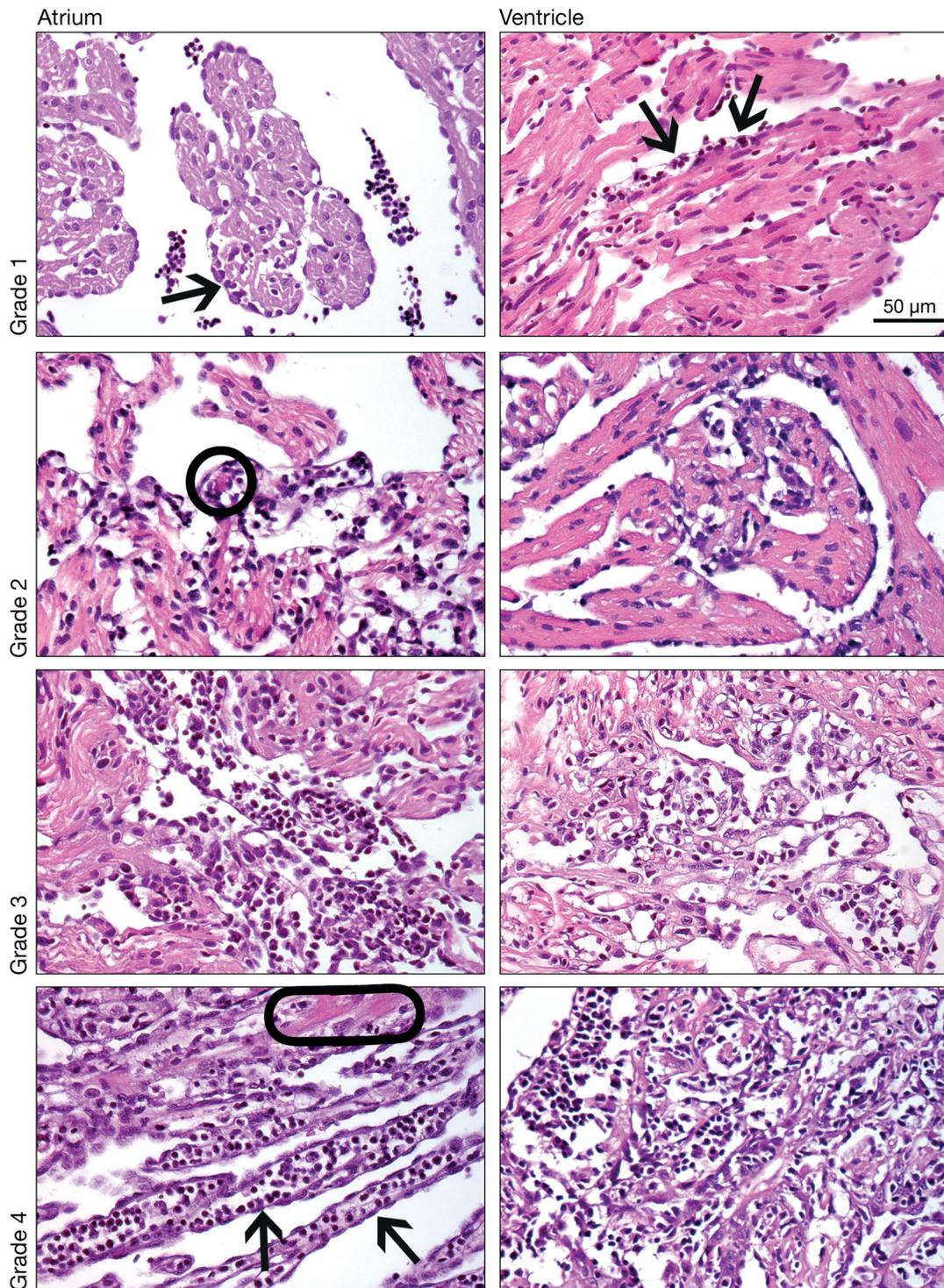


Fig. 1. *Salmo salar*. Histological classification of lesions in cardiac atrium and spongy ventricle, in accordance with the grading described in Table 1. Grade 1: arrows indicate minor inflammatory lesions consisting of sparse, focal subendocardial infiltration by mononuclear leukocytes and some degree of subendocardial vacuolisation, in both atrium and ventricle. Grade 2: several distinct lesions with small to moderately increased number of leukocytes. Myocyte degeneration and necrosis are encircled in the atrium. Grade 3: Multifocal to confluent lesions with moderate to severe leukocyte infiltration. Grade 4: Arrows indicate hypertrophic endocardial cells in the atrium forming empty tubes where almost all muscle fibres have been replaced by inflammatory cells, dominated by small mononuclear lymphocyte-like cells. Myocyte degeneration and necrosis are encircled in the atrium. Ventricular example is from a focal Grade 4 lesion. Haematoxylin and eosin staining. Scale bar (applies to all panels) = 50 µm

saged onto corresponding cells and incubated for a further week and investigated as described above.

RESULTS

Clinical signs

Only 2 fish showed clinical signs. One challenged fish was observed lying on the bottom of the tank at the first scheduled sampling and was included in the sampling. Another lethargic challenged fish was sampled 12 wk p.i.

Mortality. A total of 132 fish (26.6%) died during the experiment. Half of these fish were from Control Group 1, which experienced an IPN outbreak. In the period from 3 to 6 wk p.i., the 2 challenged groups and Control Group 1 displayed a distinct increase in mortality (15, 23 and 17 mortalities, respectively). A few weeks later (9 to 11 wk p.i.), the mortality in Control Group 1 peaked again and another 41 fish died during an IPN outbreak. The mortality of Control Group 2 followed a similar pattern (Fig. 2), but was lower, of shorter duration, and most likely related to fin and skin ulcerations (Table 2). There was no mortality in the control groups after Week 11 p.i., while an additional 12 challenged fish died sporadically throughout the study period, probably related to CMS.

Autopsy. Twelve fish from the challenged groups, the majority sampled 24 wk p.i. or later, had distended atria with or without blood clots. In every sampling from Week 24 p.i., sparse amounts of melanin-like deposits were visible in the atrium of 1 or 2 challenged fish.

Two challenged fish (12 and 24 wk p.i.) had fibrinous casts on the liver surface. From 12 wk p.i., and particularly in the last 6 samplings, a pale (9 fish), discoloured (6 fish) and/or yellowish (4 fish) liver was observed in a total of 14 challenged fish. No gross liver findings were observed in the control fish before the 2 last samplings, where 10 control fish displayed a pale (8 fish), yellowish (3 fish) and/or discoloured (6 fish) liver.

Throughout the study, most of the experimental fish suffered sparse, diffuse scale loss. In the first 24 to 27 wk, approximately half of the experimental fish also had mild to moderate skin lesions on one or both pectoral fins, with a varying degree of haemorrhage. Some of these fish had similar lesions on other fins and the tail, as well. Such

lesions were not observed after Week 24. However, scarred fins and skin were seen in previously affected fish throughout the study. Four fish in Challenged Group 1 showed mild, uni-, or bilateral central cataract at the samplings 36 and 42 wk p.i.

Mortalities from Weeks 21 (2 fish) and 33 (1 fish) all displayed similar changes: distended atria with blood clots and varying degree of haemopericardium, in addition to fibrinous casts on the liver surface, a yellowish liver and ascites.

Histopathology. The fish injected with material from diseased fish developed lesions consistent with CMS

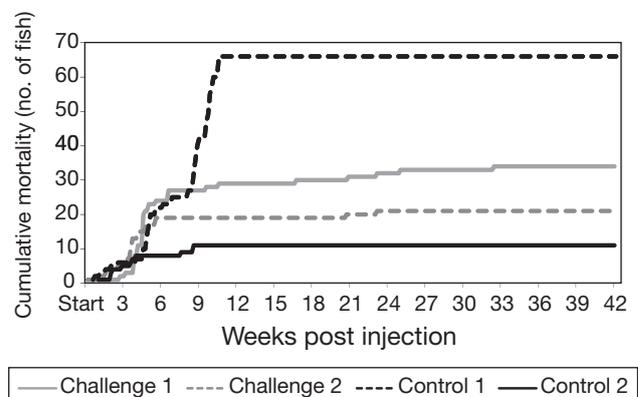


Fig. 2. *Salmo salar*. Cumulative mortality of Atlantic salmon after being intraperitoneally (i.p.) injected with tissue homogenate from Atlantic salmon diagnosed with cardiomyopathy syndrome (Challenged Groups 1 [grey solid line] and 2 [grey dotted line]) and after being i.p. injected with negative control inoculate (Control Groups 1 [black dotted line] and 2 [black solid line]). Total number of dead fish in each group to the right of the graph

Table 2. *Salmo salar*. Results of real-time RT-PCR examinations, p.i.: post-injection

Time sampled	Category of fish	No. of fish tested	IPNV+
Pre-challenge	Fish from field outbreak of CMS (6 fish), material for inoculate	6	3
Pre-challenge	Experimental fish	5	0
3 wk p.i.	Scheduled sampling	20	0
6 wk p.i.	Scheduled sampling, challenged fish with heart lesions	1	1
8.5 wk p.i.	Mortalities, Control Group 2	2	0
9–10 wk p.i.	Mortalities, Control Group 1	8	8
12 wk p.i.	Scheduled sampling	20	
	<i>Challenged fish</i>	10	8
	<i>Control Group 1</i>	5	4
	<i>Control Group 2</i>	5	0
15 wk p.i.	Histopathology + Challenged Group 1	1	1
24 wk p.i.	Scheduled sampling, incl. 5 fish from Control Group 2 only	15	
	<i>Challenged fish</i>	10	9
	<i>Control Group 2</i>	5	0
27 wk p.i.	Histopathology + Challenged Group 1	2	2

(Ferguson et al. 1990). Detailed results are presented in Figs. 3 & 4, and findings according to time are shown in Fig. 5. Focal to multi-focal inflammation became evident in the atrium 6 wk p.i., with subsequent progression to the spongy myocardium of the ventricle 9 wk p.i. The inflammation in both the atrium and ventricle was dominated by mononuclear leukocytes, in addition to necrosis of spongy myocardium and endocardium.

Early atrial lesions observed at 6 wk p.i. were mild to moderate. The number of affected fish increased from 9 wk p.i. Severity of atrial lesions peaked at 12 wk p.i., and remained at this level for the rest of the study. The most extensive atrial lesion (Grade 4) was recorded in a single fish sampled 24 wk p.i. Grade 1 to 4 atrial lesions were found in at least 60% of the challenged fish at all samplings from 9 wk p.i., except at 33 wk p.i. Melanin-like deposits as observed at autopsy were located subendocardially in atrial lesions and in degenerated atrial myocardium. In

the most severe foci, the deposits were fairly large (Fig. 6).

Mild lesions in the spongy layer of the ventricle (Grade 1) were initially detected in 3 fish with atrial changes at 9 wk p.i. The first fish with more moderate spongy layer lesions (Grade 2) was registered at 12 wk p.i., and, in the following samplings, the number of fish with Grade 2 lesions remained fairly constant. Ventricular spongy layer lesions of Grades 1 to 3 were found in at least 50% of the challenged fish at all samplings from 12 wk p.i., except at 27 and 39 wk p.i. The most severe inflammatory changes observed in this tissue were Grade 3, occurring in a few fish at 30, 33 and 42 wk p.i.

In the epicardium, a slightly increased number of leukocytes were observed in 34 of the 130 challenged fish, but in accordance with Table 1, these were graded 0. A total of 23 challenged fish displayed Grade 1 lesions in the epicardium, and 2 fish displayed Grade 2 lesions. The number of fish with epicardial changes decreased towards the end of the study.

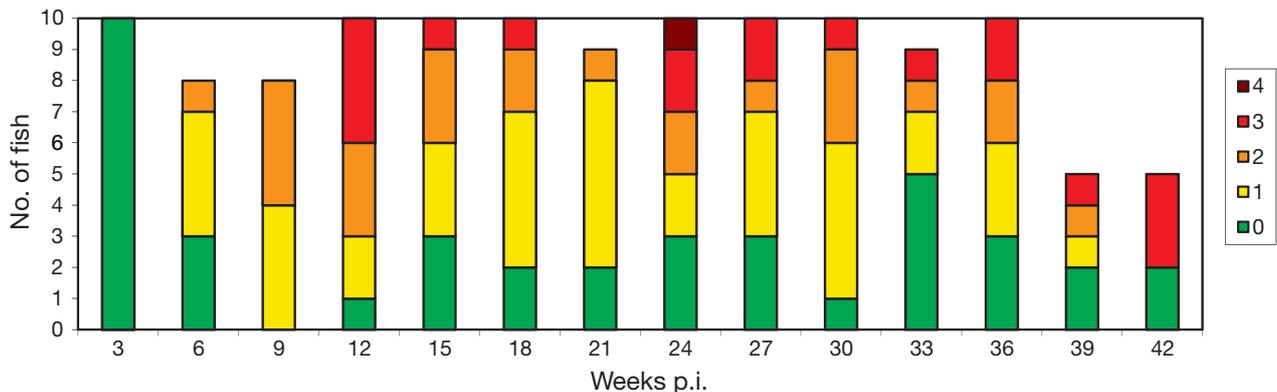


Fig. 3. *Salmo salar*. Histological findings in the atrium of the challenged fish. The lesions were classified from 0 (normal) to 4 (severe changes) according to Table 1. No or very little atrial tissue was present in the histological samples of 1 or 2 fish at 6, 9, 21 and 33 wk post-injection (p.i.) and could, therefore, not be evaluated in these fish. Due to high mortality in Challenge Group 1, the last fish in this group were sampled 36 wk p.i.

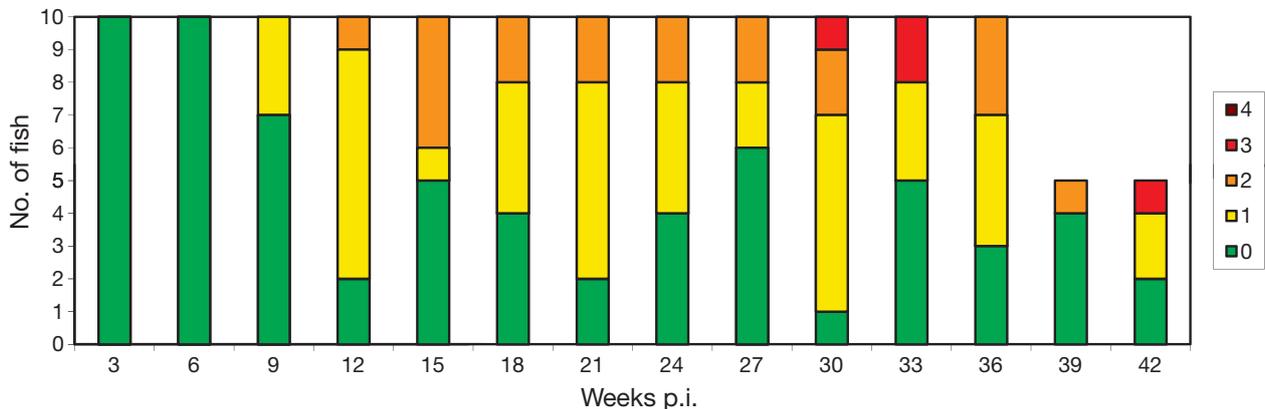


Fig. 4. *Salmo salar*. Histological findings in the spongy layer of the ventricle of the challenged fish. The lesions were classified from 0 (normal) to 4 (severe changes) according to Table 1. Due to high mortality in Challenge Group 1, the last fish in this group were sampled 36 wk post-injection (p.i.)

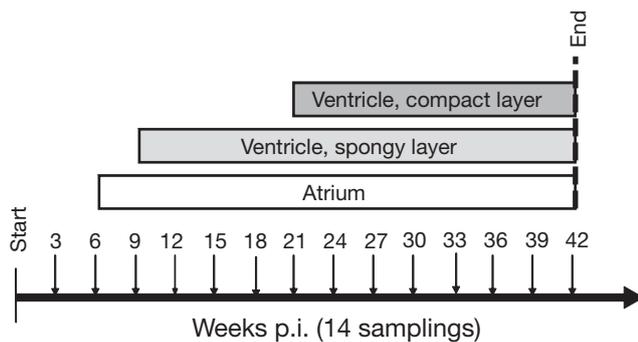


Fig. 5. *Salmo salar*. Appearance of histological cardiac lesions (as observed by light microscopy) over time post-injection (p.i.)

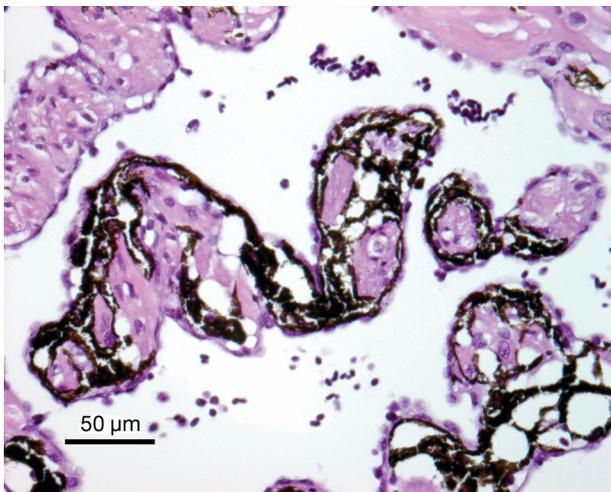


Fig. 6. *Salmo salar*. Melanin deposits in atrial lesions of experimental fish (36 wk post-injection) with a diagnosis of cardiomyopathy syndrome

Mild (Grade 1) compact layer lesions were first registered in a single fish at 18 wk p.i. and in 3 fish at 21 wk p.i. Thereafter, 1 or a few well defined foci of inflammatory cells in the compact layer, ranging in severity from Grade 1 to 2, were observed in 1 to 3 of the fish sampled at 30, 33, 36 and 42 wk p.i. All 12 fish with lesions in the compact layer also had inflammatory lesions in the spongy layer of the ventricle of identical or greater severity, while 7 of them also displayed additional focal Grade 1 or 2 lesions in the epicardium.

No CMS-like lesions were identified in the 5 fish sampled prior to challenge. None of the control fish had pathological changes in the compact layer of the ventricle, and only non-specific and sparse findings graded 0 or 1 according to Table 1 and Fig. 1 were detected in the hearts of a few fish. During the first distinct mortality period, IPN was diagnosed by histopathology and IPNV immunohistochemistry in both challenged groups and Control Group 1, but not in Control Group 2.

Microbiology

Bacteriology. Bacteria were cultivated from 8 sampled fish (4 challenged) and 1 dead fish (control). The bacterial growth was dominated by a mixed flora considered to be of little or no significance. In only 2 fish (3 wk p.i.) were bacteria, subsequently identified as *Vibrio* sp. (dead, control fish) and sparse mixed flora (sampled, control fish), isolated from both kidney and skin or fin lesions.

RRT-PCR. All examined samples were negative for nodavirus and SAV by RRT-PCR. Three of the 6 fish used for inoculation of challenged fish had low to moderate levels of IPNV (Table 2). This was also the case in all examined fish from both challenged groups and Control Group 1. During the period of high mortality, moderate to low amounts of IPNV were found in all mortalities examined from the 2 challenge groups and Control Group 1. Fish tested for IPNV in Control Group 2 were negative throughout the study.

Cell culture. No cytopathic effect was detected in any of the cell cultures at the chosen incubation temperatures.

DISCUSSION

In the present study cardiac lesions consistent with CMS were successfully transmitted to naïve Atlantic salmon *Salmo salar* post-smolts following i.p. injection of tissue homogenate from diseased fish. Both characteristics and severity of histopathological changes were reproduced in experimental fish. In addition, typical clinical signs and gross lesions were observed in some fish (Ferguson et al. 1990, Poppe & Ferguson 2006). These results indicate that CMS may be caused by an agent present in cardiac or renal tissue.

The first histopathological lesions appeared in the atrium from 6 wk p.i., while lesions in the spongy layer of the ventricle did not appear until 9 wk p.i. Generally, ventricular lesions were not seen in experimental fish without associated, and mostly more severe, atrial lesions. The explanation for this is uncertain, but the same pattern is typically seen in fish sampled from field outbreaks (T. T. Poppe unpubl. data). In such field material, atrial lesions are generally more severe than ventricular lesions, indicating a more advanced stage of the disease process in the atrium. The sequential occurrence of cardiac changes observed in the present study emphasizes the importance of sampling both atrial and ventricular tissues for histopathology. Evaluation of both tissues is a clear advantage in histopathological diagnostics, in order to differentiate between CMS and similar heart diseases.

Typical gross lesions, including enlarged atrium, fibrinous casts on the liver surface and ascites were seen in several challenged fish. These were mostly associated with histological findings of moderate to severe atrial lesions. Other typical autopsy findings in field cases of CMS indicating circulatory collapse, e.g. skin haemorrhage, raised scales and exophthalmia (Bruno & Poppe 1996), were not seen. This may be associated with the low degree of clinical disease and/or mortality in the present study. Most experimental fish showed normal swimming and feeding behaviour, in spite of severe cardiac lesions. These findings are in accordance with naturally occurring CMS, in which extensive cardiac lesions do not necessarily result in clinical disease (T. T. Poppe unpubl. data). Cardiac lesions similar to those seen in CMS have been observed in wild Atlantic salmon in Norway, but have not been associated with clinical disease (Poppe & Seierstad 2003). Several factors may influence the outcome of CMS under natural conditions. For instance, vaccination status, physiological stage, feeding regime, water temperature, oxygen levels, concurrent infections, parasite burden and other stressors may contribute to the development of clinical disease. Also, infection pressure, transmission route and factors related to pathogen exposure may be of significance. The almost simultaneous occurrence of increased mortality in all fish groups during the first weeks of this experiment could indicate exposure of the fish to unidentified environmental stressors in addition to the bacterial skin and fin infections. The markedly higher number of dead fish in 3 of 4 groups appears to be linked to the IPN diagnosis in these groups. Both the relative importance of infectious and environmental components in CMS-associated mortality and the potential for horizontal transmission should be further studied.

The experimental fish differed in at least 3 ways from the farmed fish typically affected in field outbreaks of CMS: (1) they were from a wild stock, (2) they were smaller and (3) they were unvaccinated. Experimental fish from a geographically isolated wild Atlantic salmon strain were chosen to reduce the risk of prior exposure to a possible causative agent present in the population or environment of farmed fish, and to avoid other factors associated with fish farming that may be of significance for the development of CMS. The results generated indicate that injection of tissue homogenate alone is sufficient for the development of histopathological changes typical of CMS. Additionally, the low age of experimental fish did not appear to hinder the development of cardiac lesions. Under natural conditions, CMS is commonly observed in large salmon (>2 kg) the second year in seawater, causing losses among large specimens of the population (Brun

et al. 2003). The present study showed that post-smolts were capable of developing the same type and severity of lesions as larger salmon. However, the size of the fish may partly explain the limited number of fish showing clinical signs in the present study. The heart/body volume ratio decreases with increasing fish size, and large fish may therefore have a lower cardiac capacity compared to smaller fish (Agnisola & Tota 1994, Gamperl & Farrell 2004), rendering them more vulnerable to the effects of severe cardiac lesions.

The use of unvaccinated experimental fish in the present study may have contributed to the successful transmission of CMS in young fish. At 30 to 40 g weight prior to seawater transfer, all farmed salmon in Norway are routinely vaccinated i.p. with oil-adjuvanted vaccines. These vaccines may induce both specific and non-specific immunity (Poppe & Breck 1997, Koppang et al. 2008), which could interfere with susceptibility to infections. However, little is known about the immunological processes involved in the development of CMS. In some challenged fish, melanin deposits were observed on atrial surfaces and/or subendocardially from 24 wk p.i. Such deposits have also been observed in field cases of CMS, but not as a consistent finding (C. Fritsvold pers. obs.). Melanin is suspected to be of importance in the inflammatory responses of salmonids, but its exact role or effect is not fully understood (Thorsen et al. 2006).

Although transmissibility was demonstrated in the present study, a causal agent was not identified. RRT-PCR indicated that neither nodavirus nor SAV3 contributed to the development of CMS in the present study. No cytopathic effect was found in cell cultures inoculated with tissue material from challenged fish with moderate to severe (Grade 3 to 4) atrial lesions, indicating that other known fish pathogenic viruses were not present. Likewise, bacteria known to grow on blood agar were sparse, and probably did not contribute significantly to the development of cardiac changes. In addition, any prospective causal agent present in the transmitted material would have to be resistant to gentamycin at the concentrations used.

A viral aetiology for CMS has been previously suggested (Grotmol et al. 1997). Filtration of the inoculate was deliberately not performed in the present experiment, to ensure that possible causative bacteria were not eliminated from the inoculate before challenging the fish. In future studies, filtration of homogenates prior to injection may be used to investigate the possibility of a viral aetiology. The virological procedures used were intended to rule out the presence of known viruses, and may not have been optimal for detecting unknown viruses. In the further search for an etiological, possibly viral, agent, a range of different cell lines and/or growth conditions should be tested. As CMS

appears to be a relatively slowly developing disease (>3 wk for development of initial histopathological changes in experimental fish), the relatively short incubation periods for the cell cultures may have been too short to allow for CPE to develop in the present study.

IPNV was identified by PCR in fish used in inoculate preparation, as well as in both challenged groups and 1 of the 2 control groups. This may explain why challenged fish experienced an IPN outbreak. The source of IPNV infection in 1 of the 2 control groups is unknown. Fish may have been infected by IPNV at the cultivation facility or at the research facility, as neither of them routinely disinfected their inlet water.

Postviral endo- and/or myocarditis are well known in human medicine (Eriksson & Penninger 2005). This may also be a mechanism in the development of CMS, for instance triggered by an infection with IPNV. A possible association between IPNV and CMS has previously been suggested (Brun et al. 2003). However, the presence of IPNV and the outbreak of IPN in one of the control groups did not induce CMS-like lesions in those fish. In addition, a similar experiment with CMS has recently been performed by Bruno & Noguera in Scotland, with findings parallel to those observed in the present study. IPNV was not found in that study (D. W. Bruno pers. comm.). It therefore seems unlikely that IPNV played a significant role in the development of CMS in the present study. However, an interaction between IPNV and a possible causal agent of CMS cannot be ruled out.

Focal pathological changes appeared in the compact myocardium of 8 fish from 30 wk p.i. Compact layer involvement is uncommon in field outbreaks of CMS, but has been observed in some fish with extensive epicarditis (Poppe & Ferguson 2006). Strict focal epicarditis was associated with the compact layer lesions in the present study, but these changes were only mild to moderate. Absence of lesions in pancreatic tissue and red skeletal muscle indicate that the myocardial lesions in our study were not associated with HSMI or PD. In support of this, failure to detect SAV3 appears to exclude PD. Myocarditis in the compact layer is particularly pronounced in HSMI (Kongtorp et al. 2004a), which is experimentally transmissible by a model similar to that used in the present study. The aetiology of HSMI is not yet fully understood. A possible presence of the HSMI agent in the inoculate or the experimental fish used cannot be excluded. However, only a few fish showed mild to moderate focal changes in the compact myocardium in the present study, while this tissue is severely affected in infection experiments with HSMI (Kongtorp et al. 2004b, Kongtorp & Taksdal 2009). The most common cardiac lesions in the present study were very similar to those seen in field cases of CMS, and

the development of cardiac changes also differed from experiments with HSMI. Furthermore, the time from challenge to appearance of lesions in the compact layer in the present study was considerably longer than in experimental transmission of both HSMI (Kongtorp et al. 2004b, Kongtorp & Taksdal 2009) and PD (McLoughlin et al. 1996, Christie et al. 2007).

In conclusion, cardiac changes consistent with CMS and, to a certain extent, clinical disease were transmitted to experimental fish. The results indicate that CMS may develop independently of PD and HSMI. Further studies are needed to establish the aetiology and pathogenesis of CMS and to develop disease-specific diagnostic tools.

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