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Short Communication

Cross-sectional study to investigate the presence of salmon pancreas disease virus in wild and feral fish populations in 10 lakes, Los Lagos Region, Chile

R Alegría[1,2], G Arriagada[3], S Urcelay[1] and C Hamilton-West[1]

1 Department of Preventive Veterinary Medicine, Faculty of Veterinary Science, University of Chile, Santiago, Chile
2 PhD Program in Agriculture, Forestry and Veterinary Science, University of Chile, Santiago, Chile
3 Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI, Canada

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Pancreas disease (PD) is a viral disease caused by the salmon pancreas disease virus (SPDV) and reported for the first time in 1976 in farmed Atlantic salmon *Salmo salar* L. in Scotland, (Munro *et al.* 1984); SPDV was isolated for the first time in Ireland in 1994, (Weston *et al.* 1999; McLoughlin & Graham 2007). PD has also been reported in USA, Canada, Norway (Taksdal *et al.* 2007), Ireland (Crockford *et al.* 1999), Spain, Italy and France (Raynard *et al.* 1992), among other European countries. SPDV was the first alphavirus detected in fish, leading the scientific world to incorporate it into the salmonids alphaviruses (SAV) (Weston *et al.* 2002). Currently, 6 different types of SAV have been identified. The most important types are SAV1, the aetiological agent of PD affecting Atlantic salmon, first isolated in Scotland and Ireland; SAV2, the aetiological agent of sleeping disease (SD), isolated for the first time in France, affecting rainbow trout *Oncorhynchus mykiss* (Walbaum); and SAV3, isolated only in Norway, giving it the name Norwegian salmonids alphavirus (NSAV), which affects both species and causes PD (Hodneland *et al.* 2005).

Pancreas disease outbreaks cause high economic impacts (Kilburn *et al.* 2012), which are mainly related to its high morbidity, mortality and the strong reduction in production growth rates (Norris, Foyle & Ratcliff 2008). Studies from European countries have described mortality rates ranging from 0.1 to 63% of salmon stocks (McLoughlin *et al.* 2003; Rodger & Mitchell 2007). As a result, the Chilean Fisheries Service (SERNAPESCA) has incorporated PD in the list of high-risk diseases for hydrobiological species, establishing active surveillance among other activities (Chile 2008).

Clinical presentation of PD: the disease affects both freshwater and seawater farming stages, with the following main clinical signs, in chronological order: sudden-onset anorexia, lethargy, increased presence of faeces in cages, increased mortality and decreased economic indicators (McLoughlin *et al.* 2002). Since the 1980s, the Chilean salmon industry has had a significant development, becoming one of the main salmon producers worldwide. Sanitary conditions have threatened the industry since its inception. For instance, there are endemic diseases, such as salmon rickettsial syndrome (SRS) (Lannan & Fryer 1993), infectious pancreatic necrosis (IPN) (Calleja *et al.* 2012) and high levels of infestation with sea lice

Correspondence: *C Hamilton-West, Department of Preventive Veterinary Medicine, Faculty of Veterinary Science, University of Chile, Santa Rosa 11.735, La Pintana, Santiago, Chile (e-mail: christopher.hamilton@veterinaria.uchile.cl)*

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(Yatabe *et al.* 2011; Hamilton-West *et al.* 2012). Arguably, the most significant sanitary event reported in the Chilean salmon farming industry was the infectious salmon anaemia (ISA) outbreak. ISA outbreak was officially confirmed in 2007 (Godoy *et al.* 2008), affecting approximately 50% and 65% of all the salmon farms located at Aysén and Los Lagos regions, respectively, between the years 2007 and 2009 (Bustos-Gallardo 2013).

Wild fish species and feral salmonid populations may play a role in the maintenance and spread of infectious and parasitic diseases. For instance, Snow *et al.* (2010) reported SAV RNA in internal organs of wild fish species caught in the vicinity of aquaculture activity, in areas with previous SAV history and also in remote areas from aquaculture activity within Scotland. In Chile, it was described that *Caligus rogercresseyi* (Boxshall and Bravo) was transmitted to farmed fish species by native rock cod *Eleginops maclovinus* (Valenciennes) and *Odontesthes regia* (Humboldt) (Carvajal, González & George-Nascimento 1998).

In early 2008, due to a suspicion by the producers of the introduction of PD, related to an increase in the mortality rates with clinical signs compatible with the disease, SERNAPESCA requests the investigation, to look for the presence of PD. This study set out to test for the presence of the SPDV in wild fish species and feral salmonid populations in lakes where salmon farming is carried out in Los Lagos Region, and to collect information about the potential role of the sampled species in the transmission of this disease.

An epidemiological study was performed in order to investigate the presence of SPDV, which belongs to the group of hypothesis testing studies (Cameron *et al.* 2008). These studies do not attempt to demonstrate absolute freedom from a disease or agent; instead, they seek to determine the likelihood of observing a certain number of positive individuals, using a diagnostic test, with a sample size *n*, from a sick or infected population, to a specific prevalence. If the likelihood is small, it can be declared, with a known confidence level, that the disease or the studied agent, if it is present in the population, has a prevalence lower than the prevalence established for the sample size calculation.

According to the technical requirements of the study, wild fish species and feral salmonids from 10 lakes of the Los Lagos Region (Figure 1), where salmon farming is carried out, were analysed. The numbers of salmon farming units registered (in brackets) in the selected lakes were as follows: lakes Rupanco (7), Llanquihue (15) and Chapo (6) for Continental Los Lagos Region and lakes Popetan (1), Tepuhueico (1), Cucao (1), Huillinco (5), Tarahuin (2), Natri (3) and San Antonio (1) for Insular Los Lagos Region**.** The study unit corresponds to each analysed fish.

Because it is impossible to work with the entire population of wild and feral species, the best approach is to use a representative sampling strategy. With this purpose and given that the target population size is not known, we used the following formula to determine sample size (Dohoo, Martin & Stryhn 2010):

$$n = \ln\alpha / \ln q. \quad (1)$$

where n = required sample size, α = used confidence level, q = (1 − minimum expected prevalence). Assuming a minimum expected prevalence of SPDV of 1% and setting the confidence level at 95%, the sample corresponds to 297 fish per lake.

Prior to the sampling activities, research-fishing licences were requested from the national authorities. An indirect non-probabilistic intentional sampling was performed (Cameron *et al.* 2008; Schwermer, Reding & Hadorn 2009). The diagnostic methodology used in this study was qRT-PCR. This methodology was described previously by Hodneland & Endresen (2006) as the reference SPDV diagnostic test. The National Veterinary Institute of Norway also employs it as the official diagnostic test for PD outbreaks in that country. Nevertheless, the field sensitivity (Se) and specificity (Sp) of qRT-PCR were not available during the study design and field activities (performed in 2009). Therefore, for the purpose of sample size calculation, the diagnostic test was considered as a perfect test. Later, Hall *et al.* (2012) calculated operational Se and Sp for qRT-PCR, used as a reference diagnostic technique for PD in Scotland. The Sp was estimated in 99%. However, for Se, the picture was very different with a mean of around 40%, with values varying according to the tissue tested.

Six sampling stations were established for each lake (all stations were georeferenced). The intentionality of the sampling aimed to capture apparently sick individuals in sampling stations, considering biological and environmental factors of fish and waterways (like distance to an operative salmon farm). The fishing operation was developed using static gillnets of different

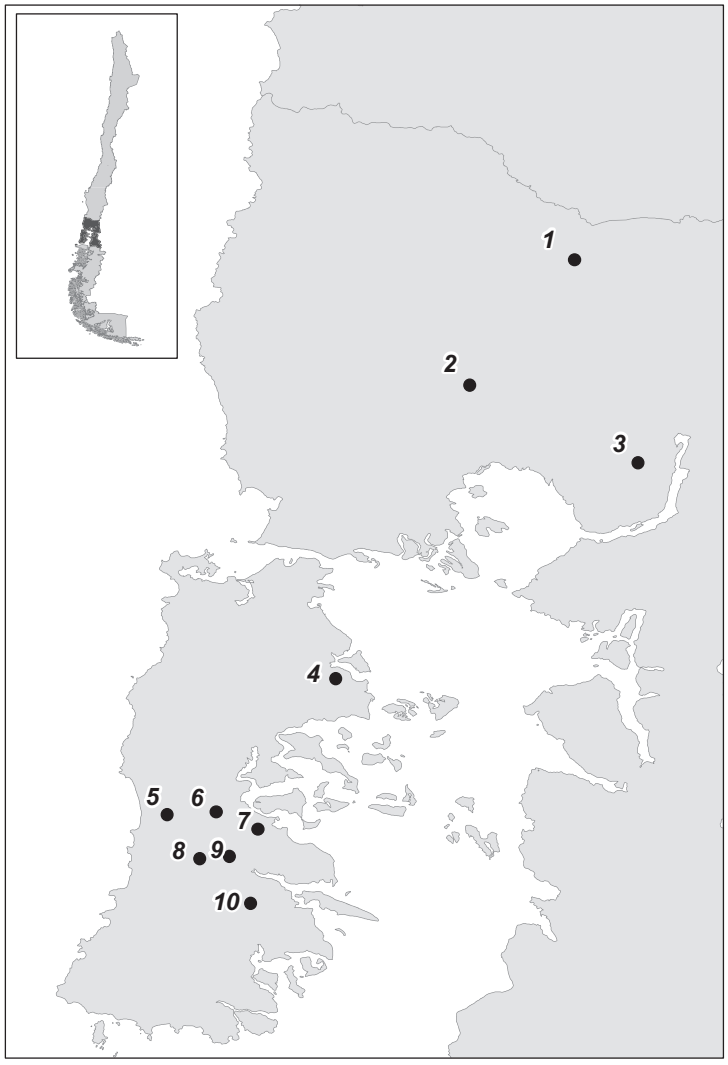

Figure 1 Lakes incorporated in the study. The Continental Los Lagos Region lakes incorporated in the study are (1) Rupanco, (2) Llanquihue and (3) Chapo. The Insular Los Lagos Region lakes incorporated in the study are (4) Popetan, (5) Cucao, (6) Huillinco, (7) Tarahuin, (8) Tepuhueico, (9) Natri and (10) San Antonio.

dimensions to allow the capture of individuals of different sizes and species.

The species, sex, size and weight were registered for each fish to assess them as potential risk factors for SPDV. Additionally, water temperature, transparency, conductivity and pH, as well as the depth of the water bodies, were measured at each sampling station.

The sampled fish were stored according to the sample station for each lake; after that, euthanasia was performed by anaesthetic overdose (benzocaine), followed by pathological examination of each individual fish. Major pathological findings, relative maturity and gut contents were registered. Finally, samples from heart, gills, kidney, liver and pancreas were collected from each fish, pooling three individuals per bag. Samples were placed in RNA-Later for preservation and transported to the laboratory.

Diagnostics were performed by ADL Diagnostic Chile Ltd. All the sampled tissues were prepared for analysis using qRT-PCR Taqman® according to a method previously described by Hodneland & Endresen (2006). To ensure the proper operation of the diagnostic test, a positive control was performed by the analysis of kidney tissue from experimentally infected Atlantic salmon with SPDV cDNA, together with a ring test, with the collaboration of Dr. David Graham from Agri-Food and Biosciences Institute (AFBI) (A. Sandoval[1], personal communication, 17 March 2010).

[1]Álvaro Sandoval, DVM. ADL DIAGNOSTIC CHILE. Pers. com. 17 March 2010.

SERNAPESCA established a fishing limit of native wild fish individuals due to legislative restrictions, indicating a maximum of five individual wild fish per lake (native species). No restrictions were defined for feral salmonids.

Sample size was achieved in six of the ten lakes included in the study (Chapo, Rupanco, Llanquihue, Tarahuín, Huillinco and Cucao). In Lake Natri, only 229 (77%) fish were captured; however, in the three remaining lakes (San Antonio, Tepuhueico and Popetan), the sample did not exceed 15% of sample size and therefore provides only a partial picture of the species inhabiting these lakes. A total of 2098 fish were sampled (71% of the total amount considered in the study design). A detail of the numbers and species sampled by lake can be observed in Table 1 and Table 2, where 2056 (98%) individuals corresponded to feral salmonids and 42 (2%) to native wild fish individuals from southern Chilean lakes.

Sex and development stage are represented in Table 3, where 1008 (48%) individuals were males and 1090 (52%) females. This trend was observed in five of the ten sampled lakes (Chapo, Rupanco, Llanquihue, Tarahuin and Cucao); for Huillinco Lake, the proportion was 38.7% of males within the sampled individuals. Only 170 individuals (8.1%) were found in the stage of reproductive maturity, while the rest of the sampled individuals corresponded to juvenile specimens or adults outside the breeding season.

A total of 707 pools corresponding to the 2098 sampled individuals from all the studied lakes were analysed. None were detected to be positive for the virus (SPDV/SAV), independent of the species; 99 pools were analysed for each lake, except for the lakes Natri, San Antonio, Tepuhueico and Popetan, where only 77, 2, 14 and 13 pools, respectively, were obtained. In the lakes where the sample size was fulfilled, it can be stated with 95% confidence level that salmon pancreas disease virus

Table 1 Number of feral fish sampled by lake and species. Los Lagos Region

Lakes	No of sampled fish	Rainbow trout *Oncorhynchus mykiss* (Walbaum)	Coho salmon *Oncorhynchus kisutch* (Walbaum)	Atlantic salmon *Salmo salar* L.
Chapo	296	264	32	–
Rupanco	292	197	91	4
Llanquihue	291	171	103	17
Tarahuin	292	241	51	–
Huillinco	292	291	1	–
Cucao	292	291	–	1
Natri	224	14	210	–
San Antonio	6	6	–	–
Tepuhueico	37	37	–	–
Popetan	34	34	–	–
Total	2056	1546	488	22

Table 2 Number of wild fish sampled by lake and species. Los Lagos Region

Lakes	No of sampled fish	Fario trout *Salmo trutta fario* (Linnaeus)	Black trout *Percichthys trucha* (Valenciennes)	Chilean silverside *Basilichthys australis* (Eigenmann)	Peladilla *Aplochiton zebra* (Jenyns)	Chilean rock cod *Eleginops maclovinus* (Valenciennes)	Peruvian rock seabass *Paralabrax humeralis* (Valenciennes)	Sardine *Sardina pilchardus* (Walbaum)
Chapo	1	–	1	–	–	–	–	–
Rupanco	5	–	1	4	–	–	–	–
Llanquihue	6	1	1	3	1	–	–	–
Tarahuin	5	–	1	4	–	–	–	–
Huillinco	5	–	–	1	1	1	1	1
Cucao	5	–	–	2	2	1	–	–
Natri	5	–	–	–	5	–	–	–
San Antonio	0	–	–	–	–	–	–	–
Tepuhueico	5	–	–	–	5	–	–	–
Popetan	5	–	–	–	5	–	–	–
Total	42	1	4	14	19	2	1	1

Table 3 Number and percentage of sampled fish by gender and development stage. Los Lagos Region

	Sex				Development stage			
Lakes	Male	%	Female	%	Juvenile/adult	%	Reproductive	%
Chapo	149	50.2	148	49.8	297	100.0	0	0.0
Rupanco	137	46.1	160	53.9	219	73.7	78	26.3
Llanquihue	174	58.6	123	41.4	296	99.7	1	0.3
Tarahuin	135	45.5	162	54.5	296	99.7	1	0.3
Huillinco	115	38.7	182	61.3	297	100.0	0	0.0
Cucao	128	43.1	169	56.9	297	100.0	0	0.0
Natri	143	62.4	86	37.6	188	82.1	41	17.9
San Antonio	4	66.7	2	33.3	2	33.3	4	66.7
Tepuhueico	9	21.4	33	78.6	11	26.2	31	73.8
Popetan	14	35.9	25	64.1	25	64.1	14	35.9
Total	1008	48.0	1090	52.0	1928	91.9	170	8.1

Table 4 Number of pools analysed by qRT-PCR salmonids alphaviruses (SAV), by lake. Los Lagos Region

Lakes	No of sampled fish	No of analysed pools	SPDV/SAV +	SPDV/SAV –
Chapo	297	99	0	99
Rupanco	297	99	0	99
Llanquihue	297	99	0	99
Tarahuin	297	99	0	99
Huillinco	297	99	0	99
Cucao	297	99	0	99
Natri*	229	77	0	77
San Antonio	6	2	0	2
Tepuhueico	42	14	0	14
Popetan	39	13	0	13
Total	2098	700	0	700

*On this lake, two pooled samples were composed by only two fish.

(SPDV/SAV), if it is present, would be at a prevalence lower than 1%; that is, it is likely that the agent is not present in the population of feral salmonids from those lakes (Cameron *et al.* 2008). In the case of the wild fish species, at least it can be stated that the sampled individuals were free of the PD agent in all tested lakes. For the remaining lakes, although the analysis yielded the same results, the critical sample size was not reached; therefore, such statements implying freedom from the agent cannot be made (Table 4).

Salmonids reproductive behaviour indicates that they perform their growth phase in the sea, to then return to the rivers or lakes where they were born. Therefore, reproduction occurs in freshwater. Wild salmonids primarily have their reproductive period in late spring and early summer in Southern Hemisphere (Fleming 1996, 2004); this agrees with and explains the results shown in Table 3, with the sampling period during late fall and winter, coinciding with low water temperatures and therefore no reproduction occurring (McLoughlin *et al.* 2003). The restriction established by national authority does not allow a detailed description of the population present in the sampled lakes (native species) and its possible interaction with feral salmonids.

Even though the required sample size was not reached in all the lakes included in the study, those lakes where it was reached have the highest concentration of salmon farms in operation and could reflect the health status of lakes in Los Lagos Region.

These epidemiological studies are considered successful, from the animal health point of view, because all the tested individuals were free of the disease or agent, and from the methodological point of view, when the calculated sample size was fulfilled. The basic elements for estimating sample size are the confidence level to be used, the Se and Sp of the diagnostic test, the establishment of a minimum prevalence detected and the probability of erroneously concluding the presence of the disease when it is not found (Thrusfield 2007; Cameron *et al.* 2008; Dohoo *et al.* 2010). It is therefore important not to overestimate or underestimate the sample size, as it could incorrectly portray economic, health and even political issues (Schwermer *et al.* 2009).

Further studies must be carried out related to diversity, population dynamics and health status of native fish populations in Chilean lakes, where salmon production is carried out, to determine their potential relation to the maintenance and spread of this and other priority diseases.

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References

Bustos-Gallardo B. (2013) The ISA crisis in Los Lagos Chile: A failure of neoliberal environmental governance? *Geoforum* **48**, 196–206.

Calleja F., Godoy M.G., Cárcamo J.G., Bandín I., Yáñez A.J., Dopazo C.P., Kibenge F.S. & Avendaño-Herrera R. (2012) Use of reverse transcription-real time polymerase chain reaction (real time RT-PCR) assays with Universal Probe Library (UPL) probes for the detection and genotyping of infectious pancreatic necrosis virus strains isolated in Chile. *Journal of Virological Methods* **183**, 80–85.

Cameron A., Gardner I., Doherr M.G. & Wagner B. (2008). Sampling Considerations in Surveys and Monitoring and Surveillance Systems. In: *Animal Disease Surveillance and Survey Systems* (ed by M.D. Salman), pp. 47–66. Iowa State Press, Ames, IA, USA.

Carvajal J., González L. & George-Nascimento M. (1998) Native sea lice (Copepoda: Caligidae) infestation of salmonids reared in netpen systems in southern Chile. *Aquaculture* **166**, 241–246.

Chile (2008). Ministerio de Economía, Fomento y Reconstrucción. Subsecretaria de Pesca. Resolución Exenta N° 2352.

Crockford T., Menzies F.D., McLoughlin M.F., Wheatley S.B. & Goodall E.A. (1999) Aspects of the epizootiology of pancreas disease in farmed Atlantic salmon Salmo salar in Ireland. *Dis Aquat Organ* **12:36**, 113–119.

Dohoo R., Martin W. & Stryhn H. (2010) *Veterinary Epidemiologic Research*. VER Inc., Prince Edward Island, Canada.

Fleming I. (1996) Reproductive strategies of Atlantic salmon: ecology and evolution. *Reviews in Fish Biology and Fisheries* **6**, 379–416.

Fleming I.A. (2004) Reproductive ecology of cultured fish in the wild. *Journal of Fish Biology* **65**, 317–317.

Godoy M.G., Aedo A., Kibenge M.J., Groman D.B., Yason C.V., Grothusen H., Lisperguer A., Calbucura M., Avendano F., Imilan M., Jarpa M. & Kibenge F.S. (2008) First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. *BMC Vet Res* **4**, 28.

Hall L.M., Wallace I.S., Munro L.A. & Murray A.G. (2012) The operational sensitivity and specificity of diagnostic testing procedures for aquatic animal infections. In: *Proceedings of the 13th International Symposia on Veterinary Epidemiology and Economics, Belgium-Netherlands. August, 2012*.

Hamilton-West C., Arriagada G., Yatabe T., Valdés P., Hervé-Claude L.P. & Urcelay S. (2012) Epidemiological description of the sea lice (Caligus rogercresseyi) situation in southern Chile in August 2007. *Prev Vet Med* **104**, 341–345.

Hodneland K. & Endresen C. (2006) Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan®). *Journal of Virological Methods* **131**, 184–192.

Hodneland K., Bratland A., Christie K.E., Endresen C. & Nylund A. (2005) New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss in Norway. *Dis Aquat Org* **66**, 113–120.

Kilburn R., Murray A.G., Hall M., Bruno D.W., Cockerill D. & Raynard R.S. (2012) Analysis of a company's production data to describe the epidemiology and persistence of pancreas disease in Atlantic salmon (Salmo salar L.) farms off Western Scotland. *Aquaculture* **368–369**, 89–94.

Lannan C.N. & Fryer J.L. (1993) Piscirickettsia salmonis, a major pathogen of salmonid fish in Chile. *Fisheries Research* **17**, 115–121.

McLoughlin M.F., Nelson R.N., Mccormick J.I., Rowley H.M. & Bryson D.B. (2002) Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, Salmo salar L. *Journal of Fish Diseases* **25**, 33–43.

McLoughlin M.F., Peeler E., Foyle K.L., Rodger H.D., O'ceallachain D. & Geoghegan F. (2003). An epidemiological investigation of the re-emergence of pancreas disease in Irish Farmed Atlantic Salmon (*Salmo Salar* L.) in 2002. In: Marine Environment and Health Series No. 14.

McLoughlin M.F. & Graham D.A. (2007) Alphavirus infections in salmon - a review. *Journal of Fish Diseases* **30**, 511–531.

Munro A., Ellis A., Mcvicar A., Mclay H. & Needham E. (1984) An exocrine pancreas disease of farmed Atlantic salmon in Scotland. *Helg Meer* **37**, 571–586.

Norris A., Foyle L. & Ratcliff J. (2008) Heritability of mortality in response to a natural pancreas disease (SPDV) challenge in Atlantic salmon, *Salmo salar* L., post-smolts on a West of Ireland sea site. *Journal of Fish Diseases* **31**, 913–920.

Raynard R., Houghton G., Munro A.L.S., Agriculture G.B.S.O. & Dept F. (1992). Pancreas Disease of Atlantic Salmon. In: *Proceedings of a European Commission Workshop, Scottish Office, Agriculture and Fisheries Department*.

Rodger H. & Mitchell S. (2007) Epidemiological observations of pancreas disease of farmed Atlantic salmon, *Salmo salar* L., in Ireland. *Journal of Fish Diseases* **30**, 157–167.

Schwermer H., Reding I. & Hadorn D.C. (2009) Risk-based sample size calculation for consecutive surveys to document freedom from animal diseases. *Prev Vet Med* **92**, 366–372.

Snow M., Black J., Matejusova I., Mcintosh R., Baretto E., Wallace I.S. & Bruno D.W. (2010) Detection of salmonid alphavirus RNA in wild marine fish: implications for the origins of salmon pancreas disease in aquaculture. *Dis Aquat Org* **91**, 177–188.

Taksdal T., Olsen A.B., Bjerkas I., Hjortaas M.J., Dannevig B.H., Graham D.A. & McLoughlin M.F. (2007) Pancreas disease in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Norway. *Journal of Fish Diseases* **30**, 545–558.

Thrusfield M. (2007) *Veterinary Epidemiology, Oxford*. Blackwell Publishing, UK.

Weston J.H., Welsh M.D., McLoughlin M.F. & Todd D. (1999) Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic Salmon, *Salmo salar* L. *Virology* **256**, 188–195.

Weston J., Villoing S., Brémont M., Castric J., Pfeffer M., Jewhurst V., McLoughlin M., Rødseth O., Christie K.E., Koumans J. & Todd D. (2002) Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *Journal of Virology* **76**, 6155–6163.

Yatabe T., Arriagada G., Hamilton-West C. & Urcelay S. (2011) Risk factor analysis for sea lice, Caligus rogercresseyi, levels in farmed salmonids in southern Chile. *Journal of Fish Diseases* **34**, 345–354.

