Research for policy support

Instrument:
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Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems

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Executive summary

Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems

Overview of project objectives

The overall goal of this project was to assess the threat of systemic iridoviruses to farmed and wild freshwater fish and amphibian wildlife in the EU.

Summary of project objectives

The project aimed to assess the threat of systemic iridoviruses (ranaviruses) to farmed and wild freshwater fish and amphibian wildlife in the EU. A major element was experimental infection trials on a range of important European species of farmed and wild freshwater fish and wild amphibians to provide data needed for the assessment. This included major farmed food species but also representative farmed ornamental fish species that are increasingly being produced in Europe and have long been speculated to be vectors of ranaviruses. Several species of wild predator fish was tested including European perch, which are highly susceptible to at least one exotic ranavirus. Representative amphibian species were also tested because wild populations appear to be seriously affected by ranaviruses. Immunological diagnostic methods for the detection of ranavirus in tissues of naturally infected species was developed to facilitate the characterization of the pathology and pathogenesis for each susceptible species and to allow rapid diagnosis in the event of a future outbreak in EU. Molecular methods were developed for the differentiation of ranavirus strains and thereby provide the means of differentiating the OIE notifiable and highly virulent isolates from the less potent isolates. Surveillance of disease in EU-produced and imported ornamental fish and pet or wild amphibians was conducted to gain knowledge of the current presence of ranavirus and indicate the potential for the aquatic pet trade to be a route of introduction and spread. Applying epidemiological principles, a risk assessment model was developed to assess the potential pathways of spread and likely impact of those viruses showing greatest virulence in the experimental trials. Recommendations were made to the Commission on future actions needed to prevent further incursion and spread of serious ranaviral diseases in the EU.

Contractors involved

P1: Technical University of Denmark, National Veterinary Institute (www.dtu.dk)
P2: Centre for Environment, Fisheries and Aquaculture Science, Weymouth (www.cefas.uk)
P3: Istituto Zooprofilattico Sperimentale delle Venezie (www.izsve.it)
P4: Finnish Food Safety Authority (www.evira.fi)
P5: Veterinary Research Institute (www.vri.cz)
P6: Friedrich Loeffler Institute (www.fli.bund.de)

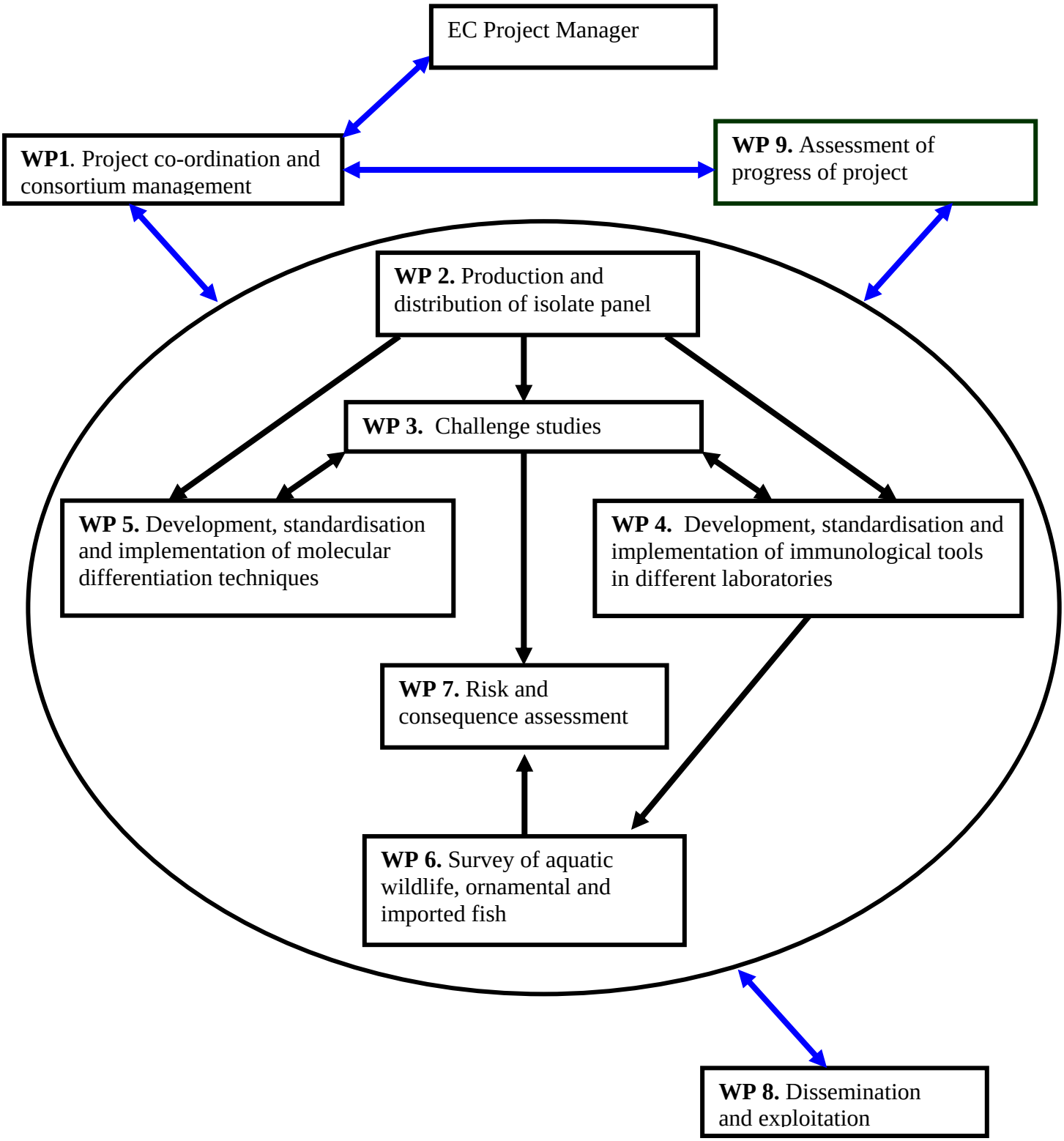

Figure 1. Graphical representation of the projects components.The overall workplan was organised in 9 workpackages covering research and innovation as well as project coordination and management.

Project objectives

The overall goal of this project was to assess the threat of systemic iridoviruses (ranaviruses) to farmed and wild freshwater fish and amphibian wildlife in the EU. This was achieved by:

1) Experimental infection trials with ranaviruses in a range of important European species of farmed and freshwater fish, including ornamental fish species and amphibians.
2) Development of diagnostic tools for rapid detection and differentiation of strains as well as a description of the pathology caused by these viruses in infected animals.
3) A risk assessment taking into account the results obtained in the experimental trials as well as the results of a laboratory and web-based survey regarding the potential presence and / or entry routes of these viruses into the EU.

Results achieved

Standardization of methods for virus isolation (WP2):

Before commencing the challenge trials and surveys, a study on the best method for isolating ranaviruses from organ material was carried out, as part of workpackage 2.
In this study, we inoculated 10 different ranavirus isolates on each of five different cell-lines. Each virus x cell-line combination was then incubated at five different temperatures for two weeks, giving a total of 250 combinations. The results from this experiment showed that all the isolates grew in all cell lines at most temperatures, but in order to be certain to detect all ranaviruses, the Bluegill fry (BF-2), Epithelio carpuloma carpio (EPC) or the Chinook salmon embryo (CHSE-214) cell-lines should be used. Additionally, the optimum temperatures for propagation were 20^{o}C, 24^{o}C and 28^{o}C whereas titres were lower at 10^{o}C and 15^{o}C. The EU-protocol requires that two cell-lines are used, either BF-2 or rainbow trout gonad (RTG-2) and either EPC or fathead minnow (FHM) cells, and that the cells are incubated at 15^{o}C. As RTG-2 and FHM were not sensitive to all ranaviruses at this temperature, the use of these two cell-lines together for detection of ranaviruses can not be recommended.

Another aim of this study was to investigate whether the EU or the OIE protocol would be the best for detection of ranaviruses, using EHNV as an example. Therefore, organ material from redfin perch (*Perca fluviatilis*) infected with EHNV was processed and inoculated on both EPC and BF-2 cells which were then treated according to each of three protocols: i) the OIE method published in 2003 of incubation at 22^{o}C for two weeks, followed by subcultivation for one week (OIE 2003), ii) incubation at 15^{o}C for two weeks, followed by subcultivation for one week and iii) the EU method of incubation at 15^{o}C for one week, followed by subcultivation for one week. The result of this experiment was that the OIE method was the best, and the EU method was the least sensitive.
The study and its results are presented in the manuscript “Propagation and isolation of ranaviruses in cell culture” by Ariel E, Nicolajsen N, Christoffersen M-B, Holopainen R, Tapiovaara H and Bang Jensen B, Aquaculture, 2009 (in press).

Susceptibility of selected European fish and amphibians to ranaviruses (WP3):

An overview of the experimental challenge studies performed can be seen in table 1.
The results of these challenges are all in the process of being published. An overview of the manuscripts where the results can be found is presented in table 2.
Pathology and pathogenesis was described for selected fish and amphibians with the aid of serological tools developed under WP4.

Table 1. overview of host species and virus combinations tested in the project. Combinations highlighted show the principal host for a particular virus.

Species tested	Temp.	EHNV	ESV	ECV	PPIV	NZ eel IV	FV3	BIV	REV-like V	GV6	DFV	RTRV	UK/ frog-life	ARC
Atlantic salmon	10°C	x	x	x	x	x	x	x						
	15°C	x	x	x	x	x	x	x						
Rainbow trout	10°C	x	x	x	x	x		x						
	20°C	x	x	x	x	x		x						
Catfish	15°C	x	x	**x**		x	x	x	x					
	25°C	x	x	**x**		x	x	x	x					
Sheatfish	15°C	x	**x**	x		x		x	x	x	x			
	25°C	x	**x**	x		x	x	x	x	x	x			
Seabass	18°C	x				x		x						
	25°C	x				x		x						
Seabream	18°C	x				x		x						
	25°C	x				x		x						
Perch	10°C	**x**	x	x	x									
	15°C	**x**				x	x	x		x				
	20°C	**x**	x	x										
	25°C	**x**				x	x	x		x		x		
Pike-perch	10°C	x	x	x	**x**		x							
	20°C	x	x	x	**x**	x	x							
Pike	10°C	x	x	x	x	x	x							
	20°C	x	x	x	x	x	x							
Common frog	15°C	x		x	x	x	x	x	x	x	x			
	20°C	x		x	x	x	x	x	x	x	x		**x**	x
Common toad	15°C	x		x			x	x	x	x	x			
	20°C	x		x			x	x	x	x	x	x		
Green frog	15°C	x		x			x		**x**	x	x			
	20°C	x		x			x	x	**x**	x	x			
Natterjack toad	15°C	x		x			x	x	x	x	x			
	20°C	x		x			x	x	x	x	x			
Smooth newt	20°C						x		x					
Pearl gourami	20°C	x	x	x				x		x	x			
	28°C	x	x	x				x		x	x			
Guppy	20°C	x	x	x			x			x	x			
	28°C	x	x	x			x			x	x			
Angelfish	20°C	x	x	x				x		x	x			
	28°C	x	x	x				x		x	x			
Zebrafish	20°C	x	x	x			x			x	x			
	28°C	x	x	x			x			x	x			
Koi carp	15°C	x	x	x			x		x	x	x			
	25°C	x	x	x			x		x	x	x			
Goldfish	15°C									x	x			
	25°C	x	x	x			x	x	x	x	x	x	X	

Table 2. Overview of manuscripts with results of the challenge trials.

Species	Manuscript	Authors	Journal	Status
Rainbow trout, Perch and Pike-perch	Susceptibility of European Farmed Freshwater Fish to a panel of ranaviruses	Bang Jensen B, Ohlemeyer S, Holopainen R, Schuetze H, Tapiovaara H, Bergmann SM and Ariel E.	Journal of Fish diseases	Submitted (August 2009)
Pike	Susceptibility of Pike *Esox lucius* to a panel of ranavirus isolates. .	Bang Jensen B., Ersbøll A.K. & Ariel E	Dis Aquat Org 2009, 83:169-179	Published
Catfish	Susceptibility of Black bullhead *Ameirus melas* to a panel of *Ranavirus* isolates	F. Gobbo, E. Cappellozza, M.R. Pastore and G. Bovo	Diseases of Aquatic Organisms	Submitted (July 2009)
Sheatfish	Susceptibility of European sheatfish to different ranaviruses	S. Ohlemeyer, S. M. Bergmann, H. Schütze.	Diseases of Aquatic Organisms	In writing
Seabass, Seabream	Susceptibility of Seabass(*Dicentrarchus labrax*) and Seabream (*sparus aurata*) to a panel of ranavirus isolates	F.Gobbo , E. Cappellozza and G. Bovo	To be determined	In writing
Common frog	Susceptibility of the European common frog, Rana temporaria, to a panel of Ranavirus isolates	Bayley A.E., Hill B.J. and Feist S.W	Diseases of Aquatic Organisms	In writing
Common toad, Green frog, Natterjack toad and smooth newt	Susceptibility of selected European amphibian species to a panel of *Ranavirus* isolates	Bayley A.E., Hill B.J. and Feist S.W	Diseases of Aquatic Organisms	In writing
Guppy, Angelfish, Zebrafish, Koi Carp and Goldfish	Experimental infection of representative ornamental fish species and carp with a panel of ranaviruses	Reschova S., K.Cinkova, B.Bang Jensen, D.Pokorova, M.Vicenova, J.Hulova, P.Kulich, T.Vesely	Journal of Fish diseases	Submitted (March 2009)
Pearl gourami	Susceptibility of pearl gourami (Trichogaster leeri) to a panel of ranaviruses	Cinkova, Reschova, Ohlemeyer, Schutze, Pokorova, Vicenova, Vesely	To be determined	In writing

Development, standardization and implementation of immunological methods for detection of ranaviruses in fish and amphibian tissues (WP4):

A polyclonal antibody for ranaviruses was developed and its specificity and sensitivity for ranaviruses was tested. The antibody cross-reacts with all the ranaviruses used in the project and is specific to ranaviruses. A protocol for immunohistochemistry on organ material from ranavirus infected animals was developed, together with a protocol on immunoflourescence on ranavirus-infected cell-layers and cryo-sections from infected organs, both using the developed antibody. The procedures were disseminated to all partners via a technical workshop for implementation in their own laboratories. Subsequent testing showed that the serological tools were implemented to a satisfactory degree in the project partners' laboratories. These procedures were then used for IHC and IFAT in the challenge trials performed, and has been shown to be very effective for identification of ranaviruses in fish and amphibian tissues and cell cultures. A manuscript describing the protocols is in preparation, and will be submitted soon (Provisional title: "Detection and identification of ranaviruses by IFAT and IHC by F.Gobbo, L.Sgro, M.Vascellari, F.Mutinelli and G.Bovo).

Molecular methods for detection and differentiation of ranaviruses (WP5):
In addition to MCP, a method to amplify the DNA-polymerase of all the virus isolates was developed, as well as a method to amplify a variable region in the Neurofilament triplet H1-like protein. For differentiation of ranaviruses, two separate restriction enzyme analysis (REA) protocols were developed: one based on the MCP gene, and another based on DNA-polymerase and Neurofilament triplet H1-like protein gene. A molecular profile of ranavirus isolates – DNA sequences of MCP, DNA polymerase and Neurofilament triplet H1-like protein was described. The methods and results are described in the manuscript: "Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes." By Holopainen R, Ohlemeyer S, Schütze H, Bergmann SM and Tapiovaara H. Diseases of Aquatic Organisms, 2009, 85(2):81-91.
The genetic stability of ranavirus isolates in different hosts was investigated by sequencing virus isolates before and after passage through different hosts. In none of the challenges were any mutations in the viruses detected, suggesting that the ranaviruses are genetically very stable, at least when passaged once through a novel host. (Details to be presented in a manuscript currently under preparation)
Molecular techniques developed in the project for differentiating and characterizing strains of ranavirus were disseminated to all partners via a technical workshop for implementation in their own laboratories. The techniques were subsequently used for detection and differentiation of viruses following challenge trials, and were in all cases successful in identifying the correct ranavirus when applied to either organ material or cell-culture.

Presence of ranaviruses in imported ornamental fish and amphibians and wild-living amphibians(WP6):
In a survey of ornamental fish imported into one of the member states, 208 consignments from 14 different countries with a worldwide representation were examined for ranaviruses in cell culture and by PCR. This survey did not reveal the presence of ranavirus in any of the samples. In a similar survey investigating imported amphibians, 150 animals from 36 separate imported consignments were tested. Amphibians positive for ranavirus were detected in 7 of these consignments, with imported animals from Ghana, Indonesia, Central America and the USA.

Another survey with the aim of investigating the presence of ranavirus in wild-living amphibians was centered around a web-based survey with public involvement. In this survey, a total of 155 samples were received from 39 separate mortality incidents in Denmark, Finland, Italy and the UK. These samples were all examined for ranavirus, and it was demonstrated that ranavirus is still present in several wild populations of amphibians in the UK, while no ranavirus was found in Italy or Finland. It was however demonstrated for the very first time in one outbreak in Denmark, as described in the article "Ranavirus in wild edible frogs (*Pelophylax* kl. *esculentus*) in Denmark." By Ariel E, Kielgast J, Svart HE, Larsen K, Tapiovaara H, Bang Jensen B and Holopainen R Diseases of Aquatic Organisms, 2009, 85:7-14.

Preliminary risk analysis of entrance and spread of one or more ranavirus in European freshwater aquaculture and wild fauna (WP7):
For the preliminary risk analysis, the OIE model for conducting a risk assessment was used to create a generic risk assessment model (RAM), and then filling information gained from other workpackages into this model, in order to obtain a preliminary risk assessment.

One of the major uncertainties when regarding ranaviruses is the lack of knowledge about susceptible species and possible vectors. This has been addressed by WP3 in the project. The presence or absence of ranaviruses in the EU has been addressed in WP6, and the opportunities for recognising disease was regarded in WP2, WP4 and WP5.
All the results thus obtained have been presented and discussed among all partners of the RANA-consortium, thereby creating the knowledge basis used by each individual when assessing and discussing the RAM.
Preceeding the RAM, a **Hazard identification** was carried out, including the panel of ranaviruses used in the project. All the viruses were considered with regards to the following assumptions:
In order for something to be a hazard it:

- Must be an OIE notifiable disease, or be identified by some other additional criteria by the importing country.
- Should not be present in the importing country - if present, the pathogenic agent should be associated with a notifiable disease, or should be subject to control or eradication measures.
- May be present in the source of material to be stocked, the exporting country, or the feeds used in the stock enhancement programme.
- Must produce adverse consequences in the importing country, including impact on related host species.
- Must be appropriate to the commodity to be imported, or to the receiving environment.

For each ranavirus isolate, these criteria were discussed. An overview of the outcome of these discussions is presented in table 3.
In the hazard identification, the following two criteria unambiguously determined if a virus could be a hazard:

- If the virus is already present in the EU, there is no need for a risk assessment of the possible entry and spread.
- It is imperative that there is a possible route of entry, i.e. importation from exporting country of a commodity which can possibly be infected or carry the virus. Therefore the hazards were re-evaluated after the pathway in figure 2 had been developed.

A very significant result from this risk assessment was the listing of ranaviruses as potential hazards or not during the hazard identification step. It was a surprise that EHNV did not classify as a potential hazard, since it has been listed by both the OIE and the EU. But as previously described, this listing has not been based on a formal risk analysis. As part of the PANDA-project (Project no. SSPE-CT-2003-502329), EHNV was ranked as a high-risk pathogen, but it was also stated that the risk of introduction was very low, since there is no pathway for EHNV to enter the EU. Furthermore, this ranking was based on rainbow trout and perch being susceptible, which they have now been proved not to be, under European conditions.

Table 3. List of possible hazards, and criteria for selection as a hazard

	Listed by the OIE or the EU?	Exotic to the EU?	Present in exporting country?	Produces adverse consequences in the EU, if introduced?	Possible route of entry present?	Hazard?
EHNV Epizootic Haematopoietic necrosis virus	Yes, by both	Yes	Present only in Australia	Depends on susceptibility of European fish & amphibians. Challenge trials reveals that pike, pike-perch, catfish, natterjack toad and possible seabream are susceptible.	No importation from Australia to the EU.	No
ESV European Sheatfish virus	No	No	Present only in the EU	Yes. Has allready caused mass mortalities in sheatfish in Germany and Finland*. Challenge trials revealed that pike and pike-perch are also susceptible. Virus could be re-isolated from infected catfish.	Allready present. Free trade within EU, since it is not listed. Angelfish, gourami, guppy and zebrafish are potential carriers**.	No
ECV European Catfish virus	No	No	Present only in the EU	Yes. Has allready caused mass mortalities in catfish in France and Italy. Pike and sheatfish also susceptible in challenge trials, positive virus re-isolation from pike-perch.	Allready present. Free trade within EU, since it is not listed. Angelfish, gourami and zebrafish are potential carriers**.	No
PPIV Pike-perch iridovirus	No	No	Present only in the EU	Depends on susceptibility of European fish & amphibians. Pike and common frog are susceptible in challenge trials, positive virus re-isolation from pike-perch.	Allready present. Free trade within EU, since it is not listed	No
NZeeIV New zealand eel virus	No	Yes	May be present in New Zealand	Depends on susceptibility of European fish & amphibians. Pike, seabass and perhaps atlantic salmon and seabream are susceptible in challenge trials, positive virus re-isolation from pike-perch.	Perhaps with trade of eels to the EU	Maybe

FV3 Frog virus 3	Yes, by the OIE (for amphibians)	No	Present in North and South America, and EU	Yes. Natural outbreaks have been seen in amphibians in the UK. European amphibians are susceptible in challenge trials. positive virus re-isolation from pike and pike-perch.	Allready present. Trade with amphibians unregulated	No
BIV Bohle iridovirus	Yes, by the OIE (for amphibians)	Yes	Present only in Australia	No natural outbreaks in the EU reported. European amphibians are susceptible in challange trials.	No importation from Australia to the EU.	No
REV-like Rana esculenta-like virus	Yes, by the OIE (for amphibians)	No	Present only in the EU	Yes. Natural outbreaks have been seen in amphibians in the UK and European amphibians are susceptible in challange trials.	Allready present. Trade with amphibians unregulated	No
RUK Ranavirus from the UK	Yes, by the OIE	No	Present only in the EU	Yes. Natural outbreaks have been seen in amphibians in the UK and European amphibians are susceptible in challange trials.	Allready present. Trade with amphibians unregulated	No
RTRV Rana tigrina ranavirus	Yes, by the OIE (for amphibians)	Yes	Present in China and Thailand	Depends on susceptibility of European fish & amphibians. No knowledge about this when RA was initiated.	Extensive unregulated trade from asia to the EU of amphibians and ornamental fish	Yes
GV6 Guppy virus	No	Yes	Present in asia (isolated in USA from asian imports)	No. Does not cause mortalities in any European species of fish or amphibians.	Extensive unregulated trade from asia to the EU of amphibians and ornamental fish. Angelfish, gourami and zebrafish are potential carriers**.	No
DFV Doctor fish virus	No	Yes	Present in asia (isolated in USA from asian imports)	No. Does not cause mortalities in any European species of fish or amphibians.	Extensive unregulated trade from asia to the EU of amphibians and ornamental fish. Angelfish and zebrafish are potential carriers**.	No

*In April 2002, 2500 pcs of 1-year old sheatfish and some 2-year old strugeon was imported from Germany.Soon after their arrival some sheatfish fell sick (36) and died and the dead fish were sent to EELA. Mortality was low (1,4%). **These ornamental fish could be infected with virus in challenge trials.

Following the hazard identification, a pathway describing the possible means of entry and spread of the ranavirus from Asia to the EU via trade with ornamental fish or amphibians was outlined (Figure 2).

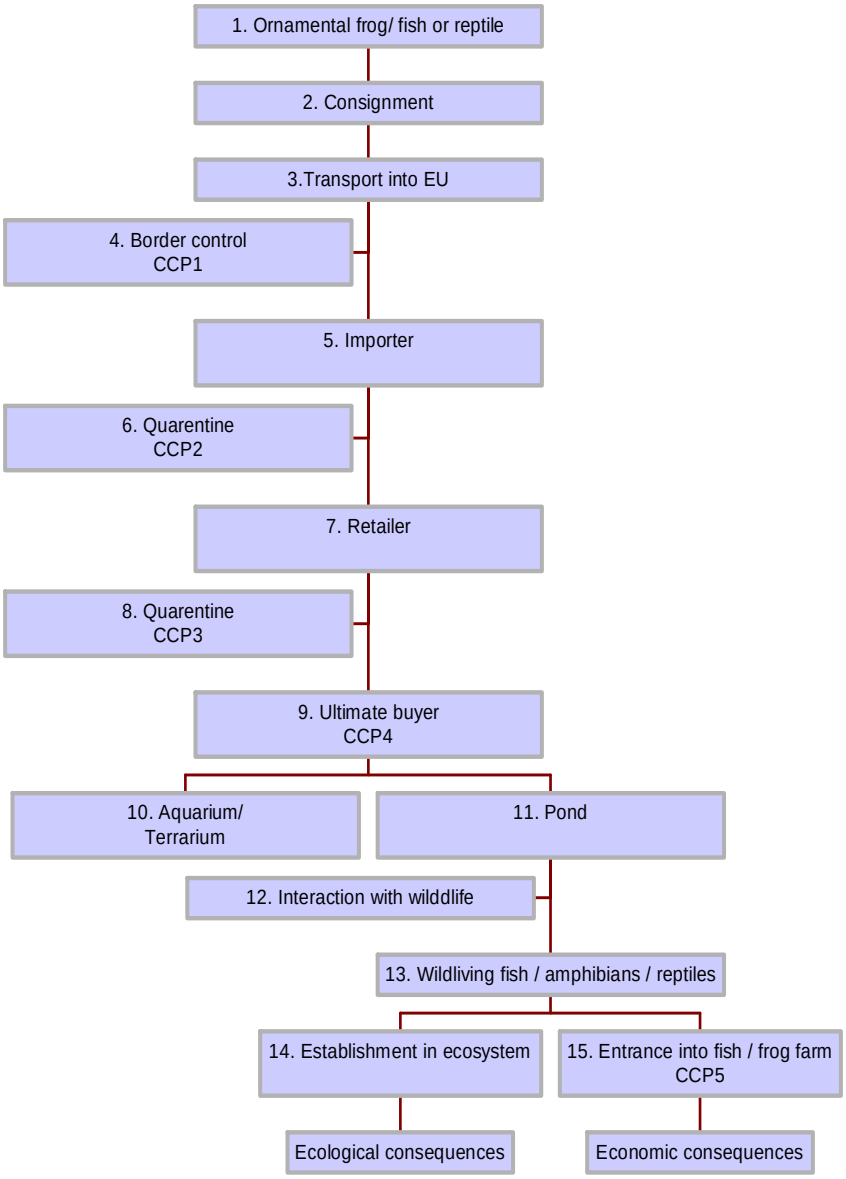

Figure 2. Pathway of introduction of exotic ranaviruses into the EU via importation of ornamental fish.

The probabilities of transmission of ranavirus to wildlife and into a fish farm are the two ultimate outcomes of the RAM.
In order to obtain some values for the quantitative RAM, the RANA-consortium and some invited experts provided thir opinions on each of the steps in the pathway. Details of the methodology and results are described in the manuscript:"A generic model for assessing the risk of introduction of ranaviruses into the EU by importation of ornamental fish and amphibians" (Bang Jensen *et al.*, in prep).

A risk estimation was made, based on the results of the risk assessment. It is based on the assumption that an imported consignment is infected with ranavirus (100% prevalence), that is, it describes what happens in **a worst-case scenario.** It was divided into the following parts:
Release assessment (likelihood of entry of an exotic ranavirus to the EU via importation of ornamental fish from Asia): *The likelihood of entry is probably very low, but any introduced ranavirus would most likely pass unnoticed.*
Exposure assessment (likelihood of susceptible animals becoming infected with ranavirus): Based on the results from the RAM, *the likelihood of wild living fauna becoming infected with ranavirus if the virus is introduced via the outlined pathway and released is low to moderate, and the likelihood of farmed fish becoming infected is very low.*
Consequence assessment (likelihood of biological, environmental or economic consequences occurring, and their likely magnitude): *Need for further research before this can be answered.*

When trying to outline a pathway for introduction of ranavirus into the EU, it quickly became apparent that the only means would be via imports of ornamental fish, amphibians and possibly reptiles. But in order for ranavirus to spread, the imported and infected animals need to get into contact with the local wild or farmed fauna. This was considered mainly to be possible when considering ornamental fish destined for outdoor ponds (goldfish and carp), and therefore the RAM focused on these. There is still the possibility that those animals that are destined for closed terrariums/aquariums might escape to the wild, but this possibility was considered negligible.
As the project moved along, infection trials revealed that Koi carp and goldfish were not susceptible to infection with ranavirus, and nor could they be carriers, even though EHNV was re-isolated from one goldfish (report D19). On the other hand, ranavirus experts from Thailand have demonstrated that goldfish can indeed be infected with RTRV, and systemic iridovirus has previously been isolated from goldfish in North America. We have no knowledge whether subclinical infected fish can transmit the virus to amphibians, and generally, much more investigation should be made into the possible transmission of ranavirus from fish to frogs and vice versa. Variation in the original strains, as seen in D16 enables a ranavirus to adapt into the new host.

In D21 it is described how no ranavirus has been found in imported ornamental fish, even though a great number and variety of species were investigated. This adds to the risk of introducing ranaviruses with ornamental fish via the outlined pathway being extremely low. On the other hand, different ranaviruses were found in frogs imported from the Americas, Asia and Africa, so it is most likely that the prevalence of ranavirus in the amphibian population is quite high. Amphibians are imported in gross amounts, and the trade is mainly unregulated. So the possibility of introducing ranavirus with imported amphibians is probably quite high. However, the probability of release of the virus into nature is very low to negligible, based on the fact that imported amphibians are kept in confined environments and do not come into contact with the wildlife.

A very obvious problem with this risk assessment is that we have no knowledge of the prevalence of ranavirus in exporting countries and the risk of a consignment being infected. This needs to be assessed, if thorough risk estimation is to be performed.

This assessment needs to be evaluated when more information is gained.
In this assessment we have considered only exotic ranaviruses. Infection with non-exotic ranaviruses is already causing mass mortalities among fish and amphibians in many EU

member states, and therefore a risk assessment of the introduction would not be appropriate. However, an exposure assessment could reveal information on the possible spread to other countries and species, and this could be followed by a consequence assessment.

The main outcome of WP7 was i) the identification of possible hazards (table 3) ii)the identification of a pathway of introduction and spread of ranaviruses into the EU (figure 2) iii) the development of a generic model to be used for assessing the risk of introducing an exotic pathogen via importation of ornamental fish and iv) identification of knowledge gaps as well as recommendations for further analytic studies needed for an optimal risk assessment of the introduction and spread of exotic ranaviruses into the EU via importation of ornamental fish.

Intentions for use and impact

The overall results of this project concluded in recommendations to the Commission on future actions needed to prevent further incursion and spread of serious ranavirus diseases in the EU, the most important of which are presented here:

1. Although the survey work demonstrated the presence of ranavirus infections in association with mortalities in wild amphibian populations in two Member States, the situation in other Member States is unknown; therefore **it is recommended** that a wider survey be carried out across the EU to determine the true geographical extent and prevalence of these infections.

3. The public involvement in the survey centred on the project's web site (www.ranavirus.net), where information on the viruses, symptoms and prevalence was presented for the public. With the completion of the RANA project, this web page has been closed down even though results of public interest are now available. **It is recommended** that funding be made available for the website be re-opened so that the results of this work can be accessible to the participants, the public.

4. **It is recommended** that the isolation of any putative ranaviruses in such a survey are confirmed in any laboratory involved by using the serological and/or molecular methods developed in this project, and genetic sequencing to determine its relatedness to other ranaviruses.

5. Whilst in this project the ranavirus infections were detected in amphibians that had died presumably from natural causes, **it is recommended** that detailed pathology studies on moribund or freshly dead animals be conducted and, if possible, also challenge experiments with the isolated virus, to confirm that these infections are actually the cause of mortalities in wild populations.

6. **It is recommended** that further research be carried out to more clearly determine the potential for natural transmission of ranaviruses between European species of fish and amphibians and the serious consequences for important farmed species and for wild populations to justify any decision to strengthen EU regulations for control and prevention of diseases caused by ranaviruses (currently directed only at exotic EHNV).

7. **It is recommended** that further studies are carried out to confirm, or otherwise, the project's finding that European strains of perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) are not susceptible to EHNV since it is currently generally assumed that the consequences of introducing this virus would be as severe as its effects

in Australia and so justifies it to be listed as an exotic disease under Directive 2006/88 with consequences for health certification of imports of susceptible and vector species.

8. The results of the molecular studies in the project have clearly demonstrated that the ranaviruses as a group are very closely related genetically. Ranaviruses are normally differentiated mainly on their different host affinities, but the results from this project have demonstrated that they may not be as host specific as has been thought, and that this differentiation is questionable. Therefore, **it is recommended** that the Commission requests EU experts to thoroughly review the current taxonomy of ranaviruses and address the question of whether it is scientifically justified to list (EHN) as the only ranavirus disease of fish in Directive 2006/88 and in the OIE Aquatic Code.

9. The OIE Diagnostic Manual recommends defined molecular methods to identify and differentiate ranaviruses but in this project one of the methods recommended by the OIE for amplification of ranaviruses was not successful in identifying Guppy virus 6 and Doctor fish virus. In contrast, the molecular methods established within this project identify and differentiate all the various ranavirus isolates in the project including the Santee Cooper ranaviruses DFV and GV6. **It is recommended** that the Commission advise the OIE to amend the chapter on EHNV on the basis of these methods.

10. **It is recommended** that additional studies (with other isolates within the genus of *ranavirus* and within the family of *Iridoviridae*) are carried out to validate and standardize these molecular methods, that the performance of such standardized methods in the EU National Reference Laboratories for fish diseases be evaluated by proficiency tests organised by a reference laboratory and that, if successful, these methods should be specified by the Commission as the required diagnostic tools for identification and differentiation of ranavirus isolates in the EU.

11. Although the susceptibility of some ornamental fish species to some of the ranaviruses in the panel was demonstrated, there was no evidence of ranavirus infections in imported consignments of ornamental fish suggesting there is very little, if any, risk of introducing exotic pathogenic ranaviruses from third-countries via the import trade in ornamental fish. Therefore, **it is recommended** that until such evidence emerges there is no case for increasing the current health certification requirements for ornamental fish under the provisions of Directive 2006/88, as far as ranavirus infections are concerned.

12. The project has provided firm evidence of exotic ranaviruses entering the EU via the import trade in ornamental amphibians (at least from Africa, Asia and the Americas) but it is unclear what the likelihood might be of their release into the aquatic ecosystem via deliberate/accidental release or escape of infected imported amphibians; therefore **it is recommended** that a survey be made for recorded cases of such amphibians being detected in the wild in the EU due to escape or accidental/deliberate release by owners.

13. Since the infected imported amphibians detected in these investigations were tropical or sub-tropical species that may not survive for long outdoors in much of the EU territory, **it is recommended** that further testing of imported consignments be carried out and be targeted at those species that can survive in a European climate. Such species are mostly imported from the USA, China and elsewhere in Asia and are often kept in outdoor terrariums or greenhouses from where escape to the natural environment is more likely than for tropical species kept indoors.

14. If sufficient evidence for significant numbers of escapes or deliberate/accidental release of imported ornamental amphibians is obtained, **it is recommended** that the European Commission considers introducing health certification requirements or other risk mitigation measures (e.g. post-importation quarantine and virus testing) for imports of live amphibians from third-countries.

15. Since some species of Chelonia (turtles, terrapins and tortoises) have been reported in the scientific literature to be infected by ranaviruses (in the case of soft-shell turtle the ranavirus is the same as that causing high mortalities in frogs in China), and large quantities for the pet trade are imported into the EU and mostly kept outdoors, **it is recommended** that any future ranavirus survey of imported amphibians be extended to include such reptile species.

16. Before considering the provisions of any possible future EU health measures for imports of amphibians (and potentially also chelonian species) from third countries, **it is recommended** that full consultation should be held with the trade organisations, many members of which are likely to be unaware of the potential disease risks to wild populations of fish and amphibians posed by the trade, to ensure they are fully informed of the research results and to enable them to play an active role in developing strategies and solutions.

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Website

The website www.ranavirus.net has been closed down with the termination of the project.

The project's current relation to the state-of-the-art

One of the main problems for the European Commission (EC) in developing policy/legislation for these ranaviral diseases is the lack of scientific data on the extent to which these viruses, or other similar ones, may be already established in European aquatic species, the possible routes of entry of exotic ranaviruses present in third countries and the likely consequences such incursions and spread could have for farmed fish and wild freshwater fauna in the EU. Furthermore, European diagnostic laboratories for fish diseases currently lack methods to rapidly detect and genetically differentiate the known ranaviruses. Even if this were possible, there is a severe lack of knowledge of the pathogenicity of these viruses for the major species of European farmed and wild freshwater fish and other aquatic fauna and of the possible pathways for their introduction and spread within the EU.

The scientific and technological objectives of this study addresses the above shortcomings in knowledge to enable a full assessment of the risk of entry of exotic ranaviruses into the EU, mechanisms for their spread and the likely consequences to freshwater fauna, and facilitates their early detection through the development of rapid and specific diagnostic tests.

Dissemination and use

Final Publications Plan

Publication title	Authors	Journal
Susceptibility of Pike *Esox lucius* (L) to a panel of ranaviruses.	Bang Jensen B, Ersbøll AK and Ariel E	Diseases of Aquatic Organisms, 83, 169-179, 2009
Susceptibility of farmed European freshwater fish to ranavirus	Bang Jensen B, Ohlemeyer S, Holopainen R, Schuetze H, Tapiovaara H, Bergmann SM and Ariel E	Journal of Fish Diseases (submitted)
A generic semi-quantitative model for assessing the risk of introducing an exotic ranavirus into the EU via imports of ornamental fish from Asia.	Bang Jensen and all partners	Diseases of Aquatic Organisms (In preparation)
Experimental infection of representative ornamental fish species and carp with a panel of ranaviruses	S.Reschova, K.Cinkova, B.Bang Jensen, D.Pokorova, M.Vicenova, J.Hulova, P.Kulich, T.Vesely	Journal of Fish Diseases (submitted)
Propagation and isolation of ranaviruses in cell culture.	Ariel, E., Nicolajsen, N., Christoffersen, M.-B., Holopainen, R., Tapiovaara, H. and Bang Jensen, B.	Aquaculture doi:10.1016/j.aquaculture.2009.05.019
Ranavirus phylogeny and differentiation based on Major capsid protein, DNA polymerase and Neurofilament triplet H1-like protein gene	Holopainen R, Ohlemayer S, Schütze H, Bergmann S and Tapiovaara H	Diseases of Aquatic Organisms 85(2):81-91, 2009
Absolute quantitation of ranavirus in cell culture and infected fish using quantitative real-time PCR.	Holopainen R, Honkanen J, Bang Jensen B, Ariel E, Tapiovaara H.	In preparation
Sequence comparison – eductase alpha and beta unit and/or otides.	Holopainen R. and Tapiovaara H.	
Survey for ranaviruses in ornamental fish.	Vesely, Cinkova, Reschova, Gobbo, Vicenova, Pokorova, Bovo	Diseases of Aquatic Organisms (Submitted)
Survey for ranaviruses in UK and imported amphibians	Hill B., Bayley A.E. and Stone D.	
Characterization of Ranavirus strains in black bullhead (*Ameiurus melas*) farmed in Italy	Bovo, Bergmann, Ohlemeyer, Tapiovara, Holopainen	
Ranavirus strains in Italian catfish farms	Bovo, Bergmann, Ohlemeyer, Tapiovara, Holopainen	
Susceptibility of black bullhead (*Ameiurus melas*) to selected ranaviruses	Bovo G. and Gobbo F.	Diseases of Aquatic Organisms (Submitted)
Sequential studies of ranavirus infection in black bullhead (*Ameiurus melas*) by IHC.	Bovo G. and Gobbo F.	
Systemic iridovirus disease in bullhead (*Ameiurus melas*) farmed in Italy	IZSVE+Evira	

Publication title	Authors	Journal
Identification of MCP-gene of GV6 and DFV by PCR and sequencing	Ohlemeyer, S., Holopainen, R., Taipovaara, H. and Schütze H.	
Diagnostic MCP PCR for ranavirus isolates	FLI+Evira	
Possible influence of host on ranavirus isolate sequence	Bergmann, Ohlemeyer, Vesely, Reschova, Schutze, Bovo, Segro, Holopainen, Tapiovara, Bayley, Hill, Bang Jensen, Ariel	
Influence of storage conditions on the infectious titre of ranaviruses	Bayley A. E. and Hill B.	
Susceptibility of selected European amphibian species to a panel of *Ranavirus* isolates.	Bayley A.E., Hill B.J. and Feist S.W	Diseases of Aquatic Organisms (In preparation)
Susceptibility of the European common frog, *Rana temporaria*, to a panel of *Ranavirus* isolates.	. Bayley A.E., Hill B.J. and Feist S.W	Diseases of Aquatic Organisms (In preparation)
Pathology of ranavirus infections in amphibians	Feist S, Bayley A.E. and Hill B.	Diseases of Aquatic Organisms
Review of the taxonomy and nomenclature of ranaviruses	All partners	
Ranavirus in wild edible frogs (*Pelophylax* kl. *esculentus*) in Denmark	Ariel E, Kielgast J, Svart HE, Larsen K, Tapiovaara H, Bang Jensen B and Holopainen R	Diseases of Aquatic Organisms, 85:7-14, 2009
Susceptibility of pearl gourami (*Trichogaster leeri*) to a panel of ranaviruses	Cinkova, Reschova, Ohlemeyer, Schutze, Pokorova, Vicenova, Vesely	In preparation
Detection of ranaviruses by an ELISA method	Cinkova, Reschova, Pokorova, Kulich, Vicenova, Vesely	Diseases of Aquatic Organisms (Submitted)
Susceptibility of European sheatfish to different ranavirus isolates	FLI	