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Evidence Project Final Report

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Project identification

1. Defra Project code: FC1189
2. Project title: Establishing a scientific basis for the length of quarantine
3. Contractor organisation(s): Centre for Environment, Fisheries & Aquaculture Science
   Barrack Road
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   Weymouth
   Dorset, DT4 8UB
4. Total Defra project costs (agreed fixed price): £ 303,052
5. Project: start date 01-04-2009
   end date 31-03-2012

6. It is Defra's intention to publish this form.

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   (a) When preparing Evidence Project Final Reports contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.

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   (b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Objective 1: Review the scientific literature on the carrier state/latent infections of fish in relation to exotic and non exotic diseases listed in Part II of Annex IV of Council directive 2006/88/EC.
Objective 2: Assess the risk of spread of disease by wild fish that are carriers of the pathogens or wild fish with latent infections with the pathogens, and the likelihood that the pathogen will be identified in those wild fish or sentinel fish following the quarantine period laid down in Draft Commission Decision SANCO/1319/2008.

Literature reviews have been carried out on the following pathogens focussing on the carrier state and the likelihood that the pathogens will be identified in wild animals or vector species in quarantine (objectives 1 and 2): Viral haemorrhagic septicaemia virus (VHSV) **;** Infectious haematopoietic necrosis virus (IHNV) **;** Spring viraemia of carp virus (SVCV) **;** Infectious salmon anaemia virus (ISAV)**;** Koi herpesvirus (KHV) **;** Epizootic haematopoietic necrosis virus (EHNV)**;** White spot syndrome virus (WSSV)**;** Yellowhead virus (YHV)**;** Taura syndrome virus (TSV)**;** *Marteilia refringens* **;** *Bonamia ostreae***;** *Bonamia exitiosa* **;** *Perkinsus marinus* **;** *Mikrocytos mackini* **;** *Aphanomyces invadans*

The review was carried out by the scientific experts at the Cefas laboratory with knowledge of the pathogens listed above. Extensive use was made of the Cefas Weymouth library database of relevant scientific literature and an extensive reprint collection, printed journals, electronic journals and photocopies of any article not held at Weymouth. **The literature reviews were included as annexes with the first year interim report submitted to Defra.**

The reviews revealed serious knowledge gaps in fundamental questions relating to the scientific basis for quarantine, such as the minimum infectious dose, to what extent survivors of the diseases became sub-clinical carriers of the pathogens, in cases in which the carrier state did exist, how long it lasted, whether or not pathogens were shed into the environment, the amount of any shedding, whether the amount of pathogen could be increased to make the pathogen easier to detect etc.

The overall conclusions are that vector species are unlikely to be a source of infection. This is because any pathogen contaminating the vector species is likely to be removed during the quarantine period and the vector species will no longer pose a threat to susceptible species. The position regarding wild fish of susceptible species (objective 2) is less straightforward, particularly because of data gaps. It is not always clear whether wild fish species, if exposed to the pathogens, are likely to become carriers of the pathogens. The circumstances in which they might shed/release the pathogen, if at all, and the amount of pathogen shed/released is often not known. As the minimum infective dose of the pathogen is not usually known it is not known whether the amount of pathogen shed/released is sufficient to cause an infection.

The time taken for patent infections to become apparent when initiated by low levels of pathogen is often not known and so the duration of the quarantine period may not be based on scientific knowledge.

A number of authors have suggested that stressing the wild animals brought into quarantine may be one way in which the pathogen may have an increased likelihood of replicating in the host and being transmitted to sentinel animals. This is because of the belief, not always backed up by experimental data, that stress or adverse environmental conditions are necessary for transmission of pathogens. However, the type of stressor to apply under quarantine conditions is unknown. Immunosuppressors may be effective, but there is a lack of data in the literature. Separate literature reviews have been carried out to collate information on pathogen detection or expression following the use of immunosuppressants and following handling stress (see objectives 4 & 5).

Specific points that emerged from the literature reviews were: Experimental transmission of *Aphanomyces invadans* in the laboratory is unusual, and there is no obvious susceptible sentinel species that would be available in the UK. The perch (*Perca fluviatilis*) is the most suitable fish to be used a sentinel species for EHNV, but some European stocks tested were refractory to the disease. Wild fish are more likely to be carriers of SVCV or KHV than of many of the other viruses and wild molluscs are likely to be infected with the mollusc parasites. The quarantine period of a minimum of 60 days for fish would appear to be appropriate for virus diseases, but there are uncertainties, and no data for *Aphanomyces invadans*. The quarantine period of 40 days for crustaceans would appear to be too short, and it is recommended that 90 days should be the minimum time. The quarantine period of 90 days for molluscs may be too short, particularly for expression of *Bonamia* sp. and *Mikrocytos mackini*. The only positive information is that should sentinel fish in quarantine be infected by the pathogen, it is highly likely that the pathogen will be identified.

Objective 3: Undertake research to determine whether alternative fish species to aquaculture species may be more appropriate as sentinel fish.

One problem with the use of sentinel animals is that susceptible animals, generally fry, of the same species may not be readily available in the UK at the time required for the quarantine period. One solution is to use susceptible fish of another species as sentinels. The stickleback has potential to be used as a sentinel species as it can survive at a range of temperatures making it suitable for testing for a range of viruses, is found naturally in both the freshwater and marine environments, and can be reared throughout its life cycle in aquaria. We have used the stickleback in previous studies as a positive control for infections with VHSV genotype IVb and can confirm its susceptibility to that virus genotype. However, the range of viruses to which the stickleback is susceptible is not fully known. As a consequence, in year 2, we commenced a study to determine the susceptibility of stickleback to serious exotic viruses: IHNV, ISAV, SVCV, tench rhabdovirus (TenRV), nervous necrosis virus (NNV, nodavirus), European sheatfish virus (ESV) (an iridovirus), VHSV (genotype I) and VHSV (genotype IVb) as positive control.
In the virus challenge trial low mortalities were seen in the tanks with sticklebacks infected with VHSV 1 and VHSV IVb and the virus was detected at low prevalence. No mortalities were seen in any other tank. It was apparent when this trial was compared to previous trials that the susceptibility of stickleback to virus infection is not consistent and their use as sentinel fish may be limited. It is possible that experimental parameters could be varied in order to achieve reproducibility, but such experimental work is unlikely to be permitted at Cefas, as the Ethical Review Committee has voiced its objection to the use of sentinel fish.

Objective 4: Undertake research using a selected pathogen or pathogens to determine the likelihood that pathogens will be identified in wild fish that are carriers of the pathogens or in wild fish with latent infections with the pathogens, or in sentinel fish following the quarantine period.

For imported salmonids, the pathogen of most concern to Defra is VHSV and, in particular, isolates belonging to sublineage VHSV IVb, which has a very wide host-range. In susceptibility trials at Cefas, tench and chub have proved susceptible to infection with VHSV IVb. A study, in year 3 of this project, on detection of virus after handling stress, confirmed that sub-clinical VHSV IVb infection could be detected in tench and chub by virus isolation or PCR methods.

There are reports that stress may be responsible for enhancing the expression of virus in subclinically-infected/carrier fish because of an immunosuppressive effect. Immunosuppressing fish during or at the end of the quarantine period could enhance the likelihood of detecting virus in carrier fish. Immunosuppression by injection of cortico-steroids has been reported to be an effective means of enhancing the isolation of bacteria from fish but there are relatively few data on the effect of immunosuppression on virus carriers. A literature search carried out in year 2 confirmed this paucity of data and concluded that this approach for carrier detection should not be pursued until 1) there are more data that show what levels of corticosteroid are effective in immunosuppressing a particular species and 2) there are more data to show that immunosuppression is effective at enhancing detection of a wide range

of viruses in a wide range of species.

A change was agreed to **Objective 5** (To undertake research using selected pathogens to determine whether the specified quarantine period is realistic) and the new objective was **'To undertake research to provide evidence to support early virus testing of quarantined fish'.**
The change was requested because of a technical constraint. The ethical review panel of the AHH division in Cefas has voiced concerns over the use of sentinel animals in quarantine studies and this makes it very difficult to investigate whether the 60 day holding period is realistic for quarantine procedures where sentinels are used. Also, a question was asked at the Defra Project Review meeting (May 2011) as to whether the quarantine period for fish could be shortened.
The change in approach to objective 5 was to undertake research to provide evidence to support early virus testing of quarantined fish. The research to provide evidence included a literature review that examined relevant data on a number of serious virus pathogens of fish. Also, experimental studies were included to compare detection of VHSV IVb in infected cyprinid fish species, in the presence or absence of handling stress.
The literature review provides good evidence to indicate that testing fish soon after their placement in a quarantine facility may provide a greater likelihood of detecting any viral pathogen infecting, or carried by, the animals. There are published studies that report that newly captured fish actively shed virus when placed into transport tanks and disease epizootics routinely occur following the stress of capture, transfer and confinement of wild cohorts in net pens or cages and in laboratory aquaria. There is also a large body of published evidence for increased cortisol responses in fish subjected to brief, acute, handling stress. So, testing fish soon after their arrival in a quarantine facility presents probably the best opportunity to detect any viral pathogens that they may be carrying.

Furthermore, a number of the viral pathogen reviews (obj 1), have addressed the quarantine of vector species and have suggested that contaminating virus may be removed during the quarantine period. So, the likelihood of the virus being detected at the end of the quarantine period is negligible. So, for vector species, tests for contaminating pathogens would be best conducted soon after the animals have been placed in the quarantine facility.

To provide further evidence for the reactivation of virus after transport and handling stress an experimental study was undertaken in year 3. The objectives of this study were to determine if virus levels are elevated in tench and chub, experimentally infected with VHSV IVb, after a brief, acute, handling stress, compared to fish that are not exposed to the stress. Also to determine if the stress can be quantified by monitoring cortisol levels in the water at different stages of the study. The results of the study indicated that virus was more frequently detected in fish exposed to stress and elevated levels of cortisol were readily detected in the water in the stress tanks. Overall, there is good evidence to support the early testing of quarantine fish soon after they have been exposed to transport related stress.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Exchange).

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The reviews revealed serious knowledge gaps in fundamental questions relating to the scientific basis for quarantine, such as the minimum infectious dose, to what extent survivors of the diseases became sub-clinical carriers of the pathogens, in cases in which the carrier state did exist, how long it lasted, whether or not pathogens were shed into the environment, the amount of any shedding, whether the amount of pathogen could be increased to make the pathogen easier to detect etc.

The overall conclusions are that vector species are unlikely to be a source of infection. This is because any pathogen contaminating the vector species is likely to be removed during the quarantine period and the vector species will no longer pose a threat to susceptible species.

The position regarding wild fish of susceptible species (objective 2) is less straightforward, particularly because of data gaps.

- It is not always clear whether wild fish species, if exposed to the pathogens, are likely to become carriers of the pathogens.
- If wild fish do become carriers it is not known whether they will shed/release the pathogen at all, or the circumstances in which they might shed/release the pathogen.
- If the pathogens are shed/released, the amount of pathogen shed/released is often not known.
- As the minimum infective dose of the pathogen is not usually known it is not known whether the amount of pathogen shed/released is sufficient to cause an infection.
- The time taken for patent infections to become apparent when initiated by low levels of pathogen is often not known and so the duration of the quarantine period may not be based on scientific knowledge.
- A number of authors have suggested that stressing the wild animals brought into quarantine may be one way in which the pathogen may have an increased likelihood of replicating in the host and being transmitted to sentinel animals. This is because of the belief, sometimes backed up by experimental data, sometimes not, that stress e.g. spawning stress, adverse environmental conditions etc is necessary for transmission of the pathogens. However, the type of stressor to apply under quarantine conditions is unknown. Immunosuppressors may be effective, but there is a lack of data in the literature.
- Separate literature reviews have been carried out to collate information on pathogen detection or expression following the use of immunosuppressants and following handling stress (see objectives 4 & 5).

Specific points that emerged from the literature reviews were:

- Experimental transmission of *Aphanomyces invadans* in the laboratory is unusual, and requires the most susceptible host species. This is the Atlantic menhaden (*Brevoortia tyrannus*), a marine fish whose geographic range is the eastern coast of North America. Hence it is unlikely that that species will be used as sentinels in the UK, and no other fish species is an obvious replacement.
- The perch (*Perca fluviatilis*) is the most suitable fish to be used a sentinel species for EHNV, but

some European stocks tested were refractory to the disease. The less susceptible rainbow trout (*Oncorhynchus mykiss*) may have to be used as a sentinel species at the high (for that species) temperature of 20°C.

- Wild fish are more likely to be carriers of SVCV or KHV than of many of the other viruses.
- Wild molluscs are likely to be infected with the mollusc parasites
- The quarantine period of a minimum of 60 days for fish would appear to be appropriate for virus diseases, but there are uncertainties, and no data for *Aphanomyces invadans*.
- The quarantine period of 40 days for crustaceans would appear to be too short, and it is recommended that 90 days should be the minimum time.
- The quarantine period of 90 days for molluscs may be too short, particularly for expression of *Bonamia* sp. And *Mikrocytos mackini*

The only positive information is that should sentinel fish in quarantine be infected by the pathogen, it is highly likely that the pathogen will be identified.

Objective 3: Undertake research to determine whether alternative fish species to aquaculture species may be more appropriate as sentinel fish.

Council Directive 2006/88/EC (specifying requirements for the prevention and control of certain disease in aquatic animals) makes provision for the importation of wild aquatic animals of species susceptible to exotic or non-exotic diseases from any source not declared disease free, to be released in a farm or mollusc farming area provided that the animals are held in quarantine before release. Draft Commission Decision SANCO/1319/2008 laying down quarantine conditions specifies a minimum period of 60 days for fish and shellfish and 90 days for molluscs. At the end of the quarantine period the animals must be tested to demonstrate absence of the relevant disease. In the case of quarantine of wild aquatic animals the use of sentinel aquaculture animals susceptible to the relevant diseases is permitted so that the animals for import are not sacrificed for testing.
One problem with the use of sentinel animals is that susceptible animals, generally fry, of the same species may not be readily available in the UK at the time required for the quarantine period e.g.: 1) the imported fish may have come to the UK at a time when no fry of that species are available ; 2) the imported fish may be of a species that is not cultivated in the UK, and obtaining fry from the wild may not be possible.
One solution to the problem of fry availability is to use susceptible fish of another species as sentinels, and it would be advantageous if that species was cultivated in this country. The stickleback has potential to be used as a sentinel species as it can survive at a range of temperatures making it suitable for testing for a range of viruses, is found naturally in both the freshwater and marine environments, and can be reared throughout its life cycle in aquaria. However, the range of viruses to which the stickleback is susceptible is not fully known. The stickleback is susceptible to viral haemorrhagic septicaemia virus (VHSV) genotypes IVa and IVb (the sticklebacks were from the marine and freshwater environments respectively) (Gagné et al., 2007; Kent et al., 1998) and an iridovirus genetically related to amphibian viruses (Mao et al., 1999). We have used the stickleback in previous studies as a positive control for infections with VHSV genotype IVb and can confirm its susceptibility to that virus genotype.

A study commenced to determine the susceptibility of stickleback to serious exotic viruses: infectious haematopoietic necrosis virus (IHNV), infectious salmon anaemia virus (ISAV), spring viraemia of carp virus (SVCV), tench rhabdovirus (TenRV), nervous necrosis virus (NNV, nodavirus), European sheatfish virus (ESV) (an iridovirus), VHSV (genotype I, European type), VHSV (genotype IVb, North American type), positive control.

The stickleback (30 per tank) were bath infected with the viruses at a concentration of 1 x 105 TCID50 ml-1, at 15°C (SVCV, TenRV, NNV and ESV) or 11°C (ISAV, IHNV and VHSV). At 3 and 6 weeks post infection 15 fish per tank were sampled: 5 for virus isolation, 5 for testing by polymerase chain reaction (PCR), and 5 for histopathology. The number of fish tested for virus isolation was reduced if there were any mortalities. However, the only mortalities that occurred were 1 fish in the TenRV tank and 1 fish in the VHSV positive control tank; both fish died shortly after the 3 week sampling (the former fish at day 28 post infection and the latter fish at day 24 post infection; the positive control fish showed typical clinical signs of VHS. Virus has only been re-isolated from fish infected with VHSV, but from both genotypes. One of the five fish from each tank sampled at day 21 was positive. The virus titres were VHSV genotype I, 3.78×10^{-5} TCID50/g, and VHSV genotype IVb, 2.58×10^{-1} TCID50/g. No virus was re-isolated from the fish that died in the TenRV tank, but was isolated from the fish that died in the VHSV positive control tank, having a titre of 1.2×10^{-8} TCID50/g. No more mortalities were seen in any tanks over the remainder of the trial. Further fish samples were taken at 42 days but all tissue samples were negative by virus isolation and quantitative PCR (qPCR) for all tanks.

The lack of mortalities in the positive control (VHSV IVb) tank was surprising, based on our previous

experience with infecting stickleback with the virus. The fish had clearly been infected with VHSV genotype IVb (and VHSV genotype I), but only at a low prevalence. The reason for that is not known, as the experimental parameters were similar to those in previous experiments in which higher mortalities occurred. This raises the question as to the susceptibility of the sticklebacks to the other viruses. Would there have been an infection under different conditions? A further consideration is the probable genetic heterogeneity of the sticklebacks used in the trial, which would contribute to a range of resistance to infection in the population. However, one conclusion from this work is that if infection of stickleback is not reproducible, the use of sticklebacks as sentinel fish is unlikely to be successful. It is possible that experimental parameters could be varied in order to achieve reproducibility, but such experimental work is unlikely to be permitted at Cefas, as the Ethical Review Committee has voiced its objection to the use of sentinel fish.

Objective 4: Undertake research using a selected pathogen or pathogens to determine the likelihood that pathogens will be identified in wild fish that are carriers of the pathogens or in wild fish with latent infections with the pathogens, or in sentinel fish following the quarantine period.

At the end of the quarantine period the animals must be tested to demonstrate absence of the relevant disease. The quarantine period may allow inapparent infections to be manifested, but alternatively it may decrease the likelihood of detecting virus disease, as often the level of virus in surviving fish decreases with the passage of time. Treating the fish in some way that increases the chance of detecting any virus in carrier fish would be an advantage, but there are limited options for such treatments.

There follows a literature review on the use of **immunosuppression to enhance the likelihood of detecting virus in carrier fish**, which is highly relevant to the quarantine procedure.

Literature review: potential of immunosuppression of fish to enhance detection of virus in carrier fish

It has been reported that stress may be responsible for enhancing the expression of virus in subclinically-infected/carrier fish, potentially by having an immunosuppressive effect (Carls et al., 1998). If that is so, immunosuppressing fish during or at the end of the quarantine period may lead to a greater chance of detecting any virus they may be carrying. Immunosuppression has been reported to be an effective means of enhancing the isolation of bacteria from fish but there are relatively few data on the effect of immunosuppression on virus carriers. Those data will be reviewed here.

Brook trout, Salvelinus fontinalis, were reared in water downstream of a population of known infectious pancreatic necrosis virus (IPNV) carrier fish (McAllister et al., 1994). At 100 days post hatch the fish were tested for virus, then at 104 days post hatch the fish were transferred to the laboratory and some were fed the glucocorticosteroid triamcinolone acetonide (also known as Kenalog) at 200 mg/kg fish in a 20% portion of the daily ration on a single occasion. After feeding, the water temperature was raised from 12°C to 18-20°C over a 1.5 h period, and then maintained at that temperature. Five and 10 days later, the fish were again fed the immunosuppressant, and the fish were tested for virus 5-7 days following the last feed with immunosuppressant. Prior to treatment with the immunosuppressant, 93.3 % of the fish were IPNV-positive with average titres of 5.4 x 10E3 plaque forming units (pfu)/ml, but after treatment, 100 % of the fish were IPNV-positive with average titres of 2.9 x 10E5 pfu/ml. The effect of both increasing temperature and immunosuppressant did significantly affect the titre of virus recovered, but the effect on virus prevalence was equivocal. Unfortunately, the experimental design did not permit separation of the effect of raising the water temperature from the effect of the immunosuppressant. Taksdal et al. (2001) tried to determine whether immunosuppression of Atlantic salmon, Salmo salar, could enhance the detection of IPNV. The fish had survived an experimental IPNV infection when they were fry and were tested for virus seven months later by virus isolation and reverse transcription polymerase chain reaction (RT-PCR) . One group of 25 fish (mean weight 15 g) were injected with 2.5 mg cortisol in 0.1 ml cocoa butter three weeks prior to sampling. That treatment had no effect on the detection of virus. The effect of immunosuppression of striped bass, Morone saxatilis, that were long-term carriers of IPNV was examined by Weschsler et al. (1986). The fish had been experimentally infected with IPNV 7 and 11 months before injection with Kenalog at 100 mg/kg body weight. The fish populations were confirmed to be 100% virus-positive by virus isolation two weeks before injection with Kenalog. Blood was taken from treated fish and was tested for the presence of IPNV and antibody against the virus. There was 30% mortality in fish injected with Kenalog, and virus was isolated from their anterior kidneys at titres similar to those obtained from fish tested before injection. Virus was not isolated from the blood of carrier fish and antibody was not detected; neither was it detected in non-treated fish. Thus the corticosteroid treatment did not affect levels of circulating virus nor antibody levels.

The effect of immunosuppression on the ability to detect carriers of herpesvirus has been investigated by van Nieuwstadt et al. (2001). The former authors purchased outwardly healthy European eels, Anguilla anguilla, ranging in weight from 150 to 200 g and maintained them in the laboratory. Groups of eels were

injected intramuscularly with 400 µg dexamethasone dissolved in 1 ml water on five consecutive days (the first treatment day was termed day 0 and the last, day 4). The presence of Herpesvirus anguillae (HVA) was determined by isolation in cell culture. Virus was detected in 6 of 40 (15%) non-injected control fish starting at day 7, whereas virus was detected in 16 of 40 (40%) fish that had been injected with the corticosteroid, starting on day 2.

There has been one trial to determine whether immunosuppression would enhance the detection of carriers of betanodavirus, but unfortunately the data are incomplete because of a freezer malfunction (Korsnes at al., 2009). Atlantic cod, Gadus morhua, average weight 100 g, were infected with nervous necrosis virus (NNV), and tested for virus by real time RT-PCR at day 25 and 130 post infection. At 150 days post infection, some fish were injected intraperitoneally (i.p.) with 0.6 ml of a 50/50 mixture of prednisolone-acetate (10 mg/ml) in coconut oil and the experiment was terminated at day 180. Samples taken at day 180 were stored at -80°C before analysis, but the freezer malfunctioned and the tissues thawed; they were still analysed for virus. Neither mortality nor clinical signs of disease were seen in any group during the 180 day trial. The distribution of NNV positive tissues at day 180 was similar to day 130, but the relative amounts of virus in spleen, kidney liver and brain increased from day 130 to day 180, and the day 180 samples were likely to be an underestimate of the amount of virus present. The amount of virus at day 180 was highest in the spleen. The authors believed this to represent an increase in virus replication following immunosuppression.

Sea bass, Dicentrarchus labrax, suffering from viral erythrocytic infection (VEI) (presumed to be caused by an as yet unidentified virus or a retrovirus) were injected i.p. with cyclophosphamide at 50 mg/kg fish. Fish injected with the corticosteroid appeared to have a greater incidence of infected erythrocytes over a 23 day period compared with non-injected fish.

Andersen et al. (2007) injected Atlantic salmon with 0.2ml of prednisolone acetate (10 mg/ ml) in an attempt to enhance the detection of salmonid alphavirus. The fish (mean weight 30 g) had been experimentally infected with the virus and were injected with the corticosteroid 171 days later. Virus was detected by real time RT-PCR, but there were only marginal differences between cortisone injected and control fish in both the numbers of fish found positive and the relative amounts of virus present.

Many of these experiments were poorly designed for investigating the effect of immunosuppression on the detection of virus from carrier fish, - for instance in some of the fish populations almost all the fish were determined to be virus positive without resorting to immunosuppression. There are also no data that indicate for those experiments in which immunosuppression does appear to help in detecting carrier fish, whether the host species or the virus, or both are the most important factor. However, some general observations can be made. Immunosuppression as a means of detecting carrier fish was successful in the case of eels infected with HVA, and possibly successful for detecting VEI in sea bass and NNV in Atlantic cod. The method was not successful in the other host-virus systems reviewed. Whether it is just a coincidence that those species are catadromous or of marine origin is not known. The exact concentration of corticosteroid used was determined empirically, and few of these reports had any data to show that the corticosteroid had had any effect on the innate or adaptive immune system, although there are now more data specifically showing those effects e.g. Skinner et al. (2010).

Conclusion: This approach for carrier detection should not be pursued until 1) there are more data that show what levels of corticosteroid are effective in immunosuppressing a particular species and 2) there are more data to show that immunosuppression is effective at enhancing detection of a wide range of viruses in a wide range of species.

Further studies were conducted in the final year of the project under objective 4.

Investigation to increase the sensitivity of virus isolation methods.

In the early days of fish virology research a small number of research groups investigated the use of cationic compounds, sometimes used in human and terrestrial virology, to enhance virus detection on cell monolayers.

Batts & Winton (1989) investigated the pre-treatment of cell monolayers with polyethylene glycol (PEG) to enhance detection of IHNV by plaque assay. When cell monolayers of EPC, FHM, CHSE-214 and BF-2 were pre-treated with 7% PEG 20,000 they produced 4 to 17-fold increases in plaque assay titres of IHNV. Plaque assay titres, in CHSE-214 cells, of VHSV, Chum salmon reovirus and Chinook salmon paramyxovirus were enhanced by pre-treatment but those of IPNV and Oncorhynchus masou virus were unaffected. Detection of IHNV in ovarian fluid samples was also enhanced by pre-treatment of EPC cells.
Leong et al. (1981) enhanced plaque formation by IHN virus 2-3 fold, in CHSE-214 cells, by pre-treatment with 5ug/ml of the polycation, polybrene (1,5-dimethyl-1,5-diazaundecamethylene-polymethobromide).

The authors had noticed a similar enhancement effect when diethyl -aminoethyl-dextran (DEAE-dextran) is substituted for polybrene. DeKinkelin et al. (1974) also enhanced pike-fry rhabdovirus infectivity in FHM cells by treatment with DEAE-dextran.

In an investigation attempted in the final year of this project, Polybrene, DEAE-dextran and PEG 20,000 were compared in their ability to enhance the infectivity of the VHSV IVb index isolate MI03.
EPC cell monolayers were pre-treated for 30 minutes with 7% PEG 20,000, 25µg/ml Polybrene or 250 µg/ml DEAE Dextran. Alternatively, Polybrene and DEAE Dextran were incorporated into the virus dilution medium at 5 µg/ml and 50 µg/ml, respectively. These treatments were then used and compared for titration of a frozen and thawed, low titre, preparation of VHSV MI03.

Some mild cytotoxicity was seen on cell monolayers treated with the higher concentration of Polybrene and moderate cytotoxicity on monolayers treated with the higher concentration of DEAE Dextran and 7% PEG. When compared to untreated cell monolayers none of the treatments appeared to enhance the infectivity of the VHSV IVb preparation. Viral cytopathic effects did not appear any earlier on treated monolayers and virus titres were the same or lower on treated, compared to untreated monolayers. Batts & Winton (1989) enhanced plaque assay titres of VHSV in CHSE-214 cells. It is possible that the treatments do not have the same enhancing effect on EPC cells or concentrations of the chemicals and treatment duration needs to be optimised. It is also possible that a greater enhancing effect may be seen when isolating virus from tissue homogenates. There will be opportunities for this work to be repeated in the new Defra projects.

Change to objective 5: To undertake research using selected pathogens to determine whether the specified quarantine period is realistic.

New Objective 5: To undertake research to provide evidence to support early virus testing of quarantined fish

In the final year of the project a change was requested to the scientific approach to objective 5 and agreed by the Defra customer. The reason for the change is because of a technical constraint. Objective 5 was to undertake research using selected pathogens to determine whether the specified quarantine period for fish in Council Directive 2006/88/EC (specifying requirements for the prevention and control of certain disease in aquatic animals) is realistic.
A long quarantine period of 60 days has been recommended as a minimum for fish. It is probable that the rationale behind the stipulation for 60 days is to provide sufficient time for any pathogen infecting the quarantined species to be transmitted to co-habiting sentinel fish. However, the ethical review panel of the AHH group in Cefas have disapproved of the use of sentinel animals in quarantine studies and this makes it very difficult to investigate whether the 60 day holding period is realistic for quarantine procedures where sentinels are used. Also, a question was asked at the Defra Project Review meeting (May 2011) as to whether the quarantine period for fish could be shortened.
The change in approach to objective 5 was to seek to determine if the length of quarantine can be substantially reduced. The research to provide evidence included a literature review (see below) that covers relevant data on all serious virus pathogens of fish. Also, experimental studies were included to compare detection of VHSV IVb, index strain MI-03, in infected cyprinid fish species, in the presence or absence of handling stress.

Objective 5: Report on evidence to support early virus testing of quarantined fish following capture and handling stress.

There is good evidence to indicate that testing fish soon after their placement in a quarantine facility may provide a greater likelihood of detecting any viral pathogen infecting, or carried by, the animals. There is a large body of published evidence for increased cortisol responses in fish subjected to brief, acute, handling stress (reviewed by Barton 2002). Greater than 10 fold increases have been reported for common carp and >90 fold for brown trout (Barton 2002). A 30-fold increase has been measured in chub exposed to netting stress (Pottinger et al. 2000). The evidence to support early testing of quarantine species is summarised below.

Vector species
Under the Defra Quarantine project, reviews of the scientific literature relevant to the quarantine conditions for susceptible species have been compiled for a number of serious pathogens. A number of these reviews, particularly for virus pathogens, have also addressed the quarantine of vector species and have suggested that contaminating virus may be removed during the quarantine period. So, the likelihood of the virus being detected at the end of the quarantine period is negligible. EFSA (Anonymous 2007) has also reported that the degree of contamination of some potential pathogen vector species (e.g. contaminated

with koi herpesvirus (KHV)) will decrease during transport. So, for vector species, tests for contaminating pathogens would be best conducted soon after the animals have been placed in the quarantine facility.

Capture and confinement of wild fish species: VHSV infection in Pacific herring

There are published studies that report that newly captured fish actively shed virus when placed into transport tanks and disease epizootics routinely occur following the stress of capture, transfer and confinement of wild cohorts in net pens or cages and in laboratory aquaria. The Pacific herring/VHSV infection system is sensitive to factors that increase the probability of infection in a single individual, including increasing the number of individuals in the tank, decreasing the rate of water exchange, increasing the proportion of highly susceptible individuals or increasing the VHSV exposure titre and duration (Hershberger et al. 1999, 2006, Kocan et al. 2001).

A number of epidemiological studies have been carried out on the wild, juvenile herring *Clupea pallasii* in Puget Sound, Washington, USA. The populations of herring in the area appear to have a low prevalence of individuals infected with VHSV (Hershberger et al. 2006). However, when captured and transported to pens or tanks the prevalence of infected fish increases, often with high mortality (Hershberger et al. 1999, 2006, Kocan et al. 2001). Stress may be a factor in the expression of disease (Hershberger et al., 2006) which was recorded as occurring within 2-3 days of capture (Kocan et al. 2001).

Hershberger et al. (1999) reported that increased prevalence of VHSV correlated with confinement of herring in net pens. Little or no virus was found in tissue samples from free-ranging, spawning herring captured from the vicinity of net pens and the prevalence of VHSV did not increase in these herring after spawning as it did in the penned herring. Kocan et al. (2001) detected VHSV in less than 1% of free-ranging , age-0 Pacific herring. However, when groups of these fish were confined in laboratory tanks they experienced mortalities that occasionally exceeded 50%, with the prevalence of VHSV reaching 100% by 14 days post capture.

Hershberger et al. (2006) reported that the capture of wild, juvenile herring and confinement in laboratory tanks resulted in outbreaks of viral hemorrhagic septicemia (VHS) and viral erythrocytic necrosis (VEN) but the timing and progression of the two diseases differed. The VHS epidemic occurred first, characterized by an initially low infection prevalence that increased quickly with confinement time, peaking at 93 to 98% after confinement for 6 d, then decreasing to negligible levels after 20 d. The VHS outbreak was followed by a VEN epidemic that, within 12 d of confinement, progressed from undetectable levels to 100% infection prevalence.

Capture and confinement of wild fish species: SVCV and TeRV infection in cyprinid fish species

Garver et al. (2007) reported the detection of SVCV in common carp, in June 2006, from Lake Ontario in Canada. The authors were uncertain as to whether the detection of SVCV in Lake Ontario represented a new introduction or whether the virus had been present and undetected in the Great Lakes for some time. Two previous samplings of common carp from Hamilton Harbour area in May and October 2005 had failed to detect the presence of SVCV or any other virus using the same methodology as used in June 2006. The authors noted that the fish were captured in June 2006, at the time of spawning, and were held in a net pen. They suggested that the combination of spawning stress and confinement may have increased fish susceptibility to SVCV infection.

Way et al. (2003) reported the isolation of a rhabdovirus, later determined to be tench rhabdovirus (TeRV), during outbreaks of disease in cyprinid fish species at a number of fishery sites in England. The virus was recovered from extracts of pooled tissues at moderate to high titres in clinically diseased fish. Thus there was strong evidence to suggest that the rhabdovirus isolated from bream and other cyprinid fish was responsible for the mortalities observed at the 6 fishery sites. They reported that preliminary laboratory transmission trials indicated that the virus was of low virulence for juvenile bream. However, in the field, mortalities were observed in fish recently introduced to the sites and suffering from handling and transport stress. Reports from the majority of the fishery sites indicated that many of the newly stocked fish were in poor condition and already suffering from fungal infections and extensive fin erosion when introduced to the site. Some external signs could be attributed to previous poor handling and transportation stress. The authors reported that it was very probable that the recently introduced fish were also experiencing suppression of their immune system at the low, early-spring water temperatures. They further suggested that the poor physiological and immunological condition of these fish very probably contributed to the severity of the disease outbreak, as has been shown in carp populations during SVC outbreaks (Fijan 1988).

Rowley et al. (2001) described the isolation and characterisation of a rhabdovirus from wild bream (Abramis brama) during a natural disease outbreak, with high mortality, in the river Bann, Northern Ireland. They reported that analysis of data from a water monitoring station on the Bann indicated a decrease in water quality coincident with the natural disease outbreak and this was considered likely to have played an important role in enhancing the severity of the outbreak.

They reported that isolation of a closely related virus from healthy bream and roach in Lough Neagh the following year was uncommon, and suggested that this was further evidence for the necessity of

appropriate trigger factors to induce clinical disease following natural infection. The isolation in NI of a similar rhabdovirus in 1984 (Adair & McLoughlin 1986) was suggested as evidence that such strains had been circulating over an extended period of time. They also suggested that the fact that the disease outbreak on the river Bann was the first described in NI during the period 1984-98, was evidence that appropriate trigger factors for disease are absent in most years.

After the outbreaks of TeRV at a number of English fishery sites in 1999, further TeRV-associated disease outbreak occurred at the Environment Agency's Calverton site in August 2004 (Internal Cefas FHI report 2004). Small-scale mortalities had been seen in a pond containing barbel and chub but high numbers of mortalities were observed in a cohort of barbel that had been transferred from the pond into an inside re-circulation system. A virus was isolated from cell cultures inoculated with tissue homogenates sampled from the barbel and subsequent sequencing confirmed the virus to be TeRV. Tests on four other cyprinid species sampled from outside ponds, and roach sampled from an inside re-circ. system, were all negative. The temperature difference between the outside pond containing the chub and barbel and the re-circ system that received the cohort of barbel was reported to be between 7 and 8°C and no gradual acclimation of the fish was attempted (A Henshaw (EA site manager), pers.comm.). This is another example of where a moderate, acute stress episode has resulted in the triggering of a disease outbreak. In this case the main trigger appears to have been the sudden rise in temperature but transport and netting may have been important contributory factors.

Stress effects in farmed fish populations: IHNV infection in rainbow trout.

In the USA, infectious haematopoietic necrosis virus (IHNV) epidemics were first observed in the Hagerman Valley in 1977 and the virus subsequently spread to become endemic throughout the valley by 1980 (Kurath et al.2003). Kurath and colleagues pointed to several features of trout farming practices that may alter the selection pressures acting on the virus. Rainbow trout have no ocean migration phase in their natural life cycle and farmed fish are held captive for their entire life span (1–2 years).
Troyer *et al*. (2000) suggested that a combination of fish culture at a relatively high constant temperature of 15°C and culture of fish at high densities and stress levels has the effect of greatly increasing the number of rounds of IHN virus replication per year on a highly intensive trout farm.
Ogut and Reno (2004) demonstrated density affects in rainbow trout fry infected with IHNV and held at a range of densities from 8 fish/L (approx 9.6 kg m^3) down to 0.012 fish L^{-1} (approx. 0.014 kg m^3). No occurrence of virus was seen at the lowest two densities (0.08 and 0.012 fish L^{-1}) but there was a positive association between increased density and IHNV prevalence in the higher densities.

Reactivation of virus in infected fish after stress events: Herpesvirus infections

An important characteristic of herpesviruses is their ability to persist in their natural hosts, including those with natural or vaccine-induced immunity (Kucuktas & Brady 1999). The virus remains dormant and non-infectious for long periods, but can be reactivated to become pathogenic with the host subsequently showing clinical signs (even mortality). The mechanism for reactivation of herpesviruses remains unknown; however, it is believed that the host's physiological state plays an important role (Roizman & Pellet 2001). The reactivation of the virus may facilitate horizontal transmission from host to other susceptible animals.
Studies on reactivation of KHV have been included in other Defra projects and investigated at the Cefas laboratory. In experimental studies, conducted by St-Hilaire et al. (2005), reactivation of the virus occurred several months after initial exposure to KHV and appeared to be temperature dependent. Reactivation of KHV occurred when the water temperature was raised to permissive levels for virus replication and was not influenced by the initial mortality associated with the virus or by co-habitation of the infected carp with naïve fish.
In transmission studies, conducted at Cefas in 2009 under the current Defra project FC1193, elevation of water temperature was used to reactivate KHV to detectable levels in wild carp. In two separate trials, common carp were obtained from two fisheries (A & B) that had experienced a clinical KHVD outbreak in 2006 and had tested antibody positive for KHV in subsequent years. Different levels of virus expression were seen in the fishery carp in the two Cefas trials. One factor that may have influenced this was the water temperature at the time of collection of the carp. Carp from site B were collected in August when the water temperature was 17°C, which was 10° higher than when the carp were collected from site A in March. The carp from site A were subjected to a gradual 16° rise in temperature and this additional stress may have provoked a greater level of virus reactivation and expression in these carp.

Research in Germany has suggested that short periods of mild to moderate netting stress may be sufficient to induce KHV reactivation (Bergmann & Kempter 2011). The reactivated virus is then detectable for 3 to 4 days after the stress period. Surviving carp that had recovered from KHVD were confined for a further 11 weeks at 20°C. To induce virus reactivation the carp were then subjected to netting stress on day 81, post infection. Gill swabs and faeces samples were then collected daily for detection of KHV by

qPCR. An increase in KHV concentration of up to 1000 KHV genomic equivalents was detected over a 3 day period post-netting but this decreased on day 4 and no KHV DNA was detected in samples taken on day 10. These studies were carried out on carp that had been persistently infected with KHV for 2 or 3 months. More work is needed to determine if herpesvirus can be reactivated from long-term virus carriers by exposure to short stress episodes and to determine the most efficient stressors or combination of stressors.

The effect of stress events on herpesvirus expression may vary in impact depending on the virus and the type of stressor employed. Davis et al. (2002) developed a quantitative bioassay employing immersion exposure for the infection of channel catfish (*Ictalurus punctatus*) with the protozoan parasite *Ichthyophthirius multifiliis*, commonly referred to as ich. This bioassay as well as waterborne challenge of channel catfish with channel catfish virus (CCV) was used to investigate the effect of confinement stress on the sensitivity of the fish to exposure of these pathogens. Infestation by ich was shown to be proportional to the density of infective theronts in the exposure tank and low-water crowding stress was shown to increase susceptibility of catfish to infection. Mortality from CCV was related to the virus exposure dose; however, low-water crowding stress did not affect mortality. The authors suggested that the increased susceptibility, due to crowding stress of naıve channel catfish to I. multifiliis but not to CCV, was because of a difference in the defence mechanisms. They suggested that stress-induced increased susceptibility to I. multifiliis may be due to a suppression of an innate protection mechanism. The lack of effect of stress on CCV mortality was explained by protection afforded by an inducible system which was not affected by the stressor. Alternatively, the lethal effects of the virus may have been too fast for the stress to change susceptibility in fish exposed to CCV for the first time.

Assessment of the most effective stressors

The stress-related effects of short-term holding of fish are influenced by water quality, confinement density, frequency of handling and holding container design. Investigators have reported that collecting, handling, sorting, holding, and transporting are routine practices that can have significant effects on fish physiology and survival (Portz et al. 2006). High fish densities in holding containers have been noted as the most common problem throughout aquaculture facilities and in live-fish transfers. Furthermore, the holding container design may also compromise the survival and immune function by affecting water quality, density and confinement, and aggressive interactions. Fishes held for relatively short durations are also influenced by negative interactions, associated with intra-specific and inter-specific competition, cannibalism, predation, and hierarchical conflict. These interactions can be lethal (i.e., predation) or may act as a vector for pathogens to enter (i.e., bites and wounds). Most importantly, all of the above factors produce stress in captive fish that commonly results in immuno-suppression and increases the likelihood of pathogen re-activation in a cohort of fish subjected to a short-period of confinement.

Fagerlund et al. (1981) carried out studies of juvenile coho salmon (Oncorhynchus kisutch) reared for 12 months at four stocking densities in rectangular concrete ponds. They found that increasing fish density was associated with significant decreases in weight, length, condition factor, and food conversion efficiency; elevated body water content; reduced fat and protein contents; and increased mortality (which did not exceed 3%). They concluded that crowding stress particularly affects small salmon.

A study by Einarsdottir and Nilssen (1996) examined the Atlantic salmon (*Salmo salar*) stress response by measuring plasma cortisol, glucose and chloride in fish after water level reduction within rearing tanks. Maximum plasma cortisol levels were observed 20 min after application of the stressor and levels had declined to control levels 24 h later. The plasma glucose and chloride concentrations did not change significantly in either the stress or control groups of salmon. They then repeatedly subjected a group of salmon to the same stressor every third day. After the fifth exposure to the stressor, analysis of blood parameters showed that cortisol levels reached a lower level than that seen after the first exposure and had declined to pre-stress levels within 2 h. Plasma glucose and chloride concentrations did not change significantly in this second experiment. They concluded that their test revealed an acute primary response in Atlantic salmon without any apparent harmful secondary responses. They also suggested that this may serve as a standardized reference stressor using other fish groups under comparable conditions.

In a study on the effects of chronic elevation of plasma cortisol on stress responses in salmonids, Pickering & Pottinger (1989) reported that basal levels of plasma cortisol in unstressed salmonid fish are normally in the range 0–5 ng ml^{-1}. In their study, acute stress such as handling or 1 h confinement caused a temporary elevation of the plasma cortisol levels of both brown trout,*Salmo trutta* L., and rainbow trout,*Salmo gairdneri* Richardson, in the range 40–200 ng ml^{-1} with a return to basal levels within 24–48 h. The extent of the cortisol elevation in response to an acute stress was dependent upon both the species and strain of trout. Chronic stresses, such as prolonged confinement or crowding, resulted in an elevation of plasma cortisol levels to approximately 10 ng ml^{-1} and blood cortisol levels remained elevated for periods of up to 4 weeks before acclimation finally occurred. They showed, by means of intraperitoneal implantation of cortisol, that chronic elevation of plasma cortisol levels in brown trout results in a dose-

dependent increase in mortality due to common bacterial and fungal diseases. Furthermore, this effect is apparent at plasma cortisol levels as low as 10 ng ml^{-1}, levels below those often reported as being representative of 'unstressed' fish.

Response to handling stress

Fishes display a wide variation in their physiological responses to stress, which is clearly evident in the plasma corticosteroid changes, chiefly cortisol in bony fishes, that occur following a stressful event. The characteristic elevation in circulating cortisol during the first hour after an acute disturbance can vary by more than two orders of magnitude among species and genetic history appears to account for much of this interspecific variation (Barton 2002).

There is a large body of published evidence for increased cortisol responses in fish subjected to brief, acute, handling stress (reviewed by Barton 2002). Greater than 10 fold increases have been reported for common carp and >90 fold for brown trout (Barton 2002). A 30-fold increase has been measured in chub exposed to netting stress (Pottinger et al. 2000).

Barton (2002) compared stress responses to brief, acute stress reported in 11 different fish species. Plasma cortisol concentrations were measured before, and 1h after, the fish were subjected to an identical 30s aerial emersion. Increased cortisol responses ranged from as low as a 1.3-fold increase in Pallid sturgeon (*Scaphirhynchus albus)* to a 94-fold increase recorded for Brown trout (*Salmo trutta*). Cortisol increases in other species were recorded as follows: Common carp (*Cyprinus carpio*) 11-fold; Walleye (*Stizostedion vitreum*) 21-fold; Brook trout (*Salvelinus fontinalis*) 21-fold; Arctic grayling (*Thymallus arcticus*) 24-fold; Yellow Perch (*Perca flavescens*) 25-fold; Rainbow trout (*Oncorhynchus mykiss*) 25-fold and Lake trout (*Salvelinus namaycush*) 46-fold.

Repeated exposure to short stress events

Repeated exposures to mild (brief, acute) stressors can desensitize fish and attenuate the neuroendocrine and metabolic responses to subsequent exposure to stressors (reviewed in Barton 2002). For example, juvenile rainbow trout were subjected to one of three different brief handling stressors once a day for 10 wk and at the end of that time, their response to acute handling was measured. The response of plasma cortisol was about half of that observed in naive, previously unstressed fish indicating possible desensitization of the cortisol-release pathway to the repeated disturbances. A matching and significant decline in the response of plasma glucose in the treatment group, which implies the involvement of the catecholamine response, suggests a general habituation to the repeated stressor (Barton 2002).

Stress response in marine fish species

Thomas & Robertson (1991) investigated plasma cortisol and glucose stress responses to several common aquaculture procedures in hatchery-reared juvenile red drum (*Sciaenops ocellatus*). The magnitude of the cortisol and glucose stress responses to capture by dip net and transfer to another tank was related to the duration of net restraint and exposure to air. Rapid transfer of fish with only 5 s exposure to air did not elicit a cortisol stress response, whereas plasma cortisol titres increased five-fold after restraint and exposure to air for two minutes. Prior anaesthesia partially blocked the response to the 2-min transfer stressor. Lowering the water level in the experimental tanks, followed by anesthetization of the fish and then slowly refilling the tanks caused a marked increase in plasma cortisol concentrations. The authors concluded from these studies that, the physiological disturbance of red drum during routine aquaculture procedures can be reduced by selecting appropriate handling and anaesthetic treatments.

As with freshwater fish species, a good deal of variation is seen in corticosteroid stress responses in marine fish species. Atlantic cod (*Gadus morhua*), is an example of a species that exhibits a low corticosteroid response to acute stress. Plasma cortisol has been measured at a low peak increase of15 ng/ml after handling (Hemre *et al.*,1991). At the high end of the response range, Maule *et al.* (1988) during their physiological monitoring studies of migrating juvenile chinook salmon (*Oncorhynchus tshawytscha*) found that peak post-disturbance cortisol concentrations often reached 400 ng/ ml during and after transport.

Objectives 4 & 5: Detection of virus in experimentally infected carrier fish after handling stress

The aim of this experimental study was to provide evidence that the sensitivity of diagnostic tests might be improved by testing imported animals soon after their arrival at a quarantine facility. The likelihood is that virus infecting the fish will be reactivated after a brief period of acute transport and handling stress. The objectives of this study were to determine if virus levels are elevated in VHSV-infected cyprinids after a brief, acute, handling stress, compared to fish that are not exposed to the stress. Also to determine if the stress can be quantified by monitoring cortisol levels in the water at different stages of the study.

As mentioned previously in the literature review, for imported salmonids, the pathogen of most concern to Defra is VHSV and, in particular, isolates belonging to sublineage VHSV IVb. This serotype has a wide host-range but experimental studies under the Defra project on Pathogen Characterisation (F1188) and

elsewhere (Kim & Faisal 2010a & b) have reported large differences in the comparative susceptibilities of different fish species to the IVb index strain MI03.

In previous trials at Cefas, nine fish species have been challenged by bath infection. Mortality was only seen in three-spined stickleback (*Gasterosteus aculeatus*) infected with VHSV IVb isolate MI03. Rainbow trout, gudgeon, rudd, barbel and orfe were refractory to infection with the virus and perch showed a transient pathology. The virus was re-isolated from stickleback but not from any of the other species. The two remaining species, tench (*Tinca tinca*) and chub (*Leuciscus cephalus*), did not show disease pathology or suffer mortalities, but virus was re-isolated from these fish. Compared to the titres of virus re-isolated from stickleback the titres of virus re-isolated from tench were low (80 - 250 TCID50/g visceral extract) but, at 28 days post infection (dpi), 5 of 5 tench sampled were positive for VHSV MI03. The MI03 isolate was re-isolated from 1 of 5 chub at 14 dpi and 1 of 4 chub at 28 dpi with titres of 55 and 1.76 $x10^{-3}$ TCID50/g visceral extract respectively. Tench and chub appear to be good candidates for this type of investigation as there is evidence to indicate that the virus is persisting in these cyprinid species, at different levels of prevalence, for a number of weeks and may be replicating in the tissues.

There is a large body of published evidence for increased cortisol responses in fish subjected to brief, acute, handling stress (reviewed by Barton 2002). Greater than 10 fold increases have been reported for common carp and >90 fold for brown trout (Barton 2002). In the species included in this study, a 30-fold increase has been measured in chub exposed to netting stress (Pottinger et al. 2000), but similar data for acute stress in tench is not available.

In this study the chub and tench (15 fish in 15L tank water) were infected by immersion with 10^5 TCID50/ml of VHSV MI03 for 4 hours at 10-12°C. At two time-points during the study, all of the fish in the stress tanks were exposed to a brief netting stress where they were held out of the water in a net for 90 seconds. The response to the stress was monitored by measuring cortisol levels in water samples both before and after application of the netting stress. The 90 second holding time is a common, standard, acute stressor and 1 litre water samples were taken immediately before and then 2 hours after the acute stress episode, when cortisol in the water is at peak levels. A water sample was also taken from the inflow water to the tanks as a reference negative control.

The fish were exposed to the netting stress at 14 days and 21 days post-infection and 5 fish were sampled from each tank, for virus detection and isolation, 2 days after each stress event. The water samples were collected and frozen (at -20°C) for later cortisol analysis. Brain, kidney spleen and heart tissues and a portion of liver tissue was dissected from the sampled tench and chub and subjected to virological analysis by virus isolation and by nested PCR.

The results of the trial were as follows. Three days after the 1st stress exposure and 1 day after the first sampling, one tench mortality was seen in 09-39 (stress tank). Virus was re-isolated at a titre of 5.6 x 10^6 TCID50/ml. No further mortalities were seen during the 5 weeks of the trial.
From the two rounds of sampling for virological examination, VHSV was isolated, or detected by PCR, from 3 chub and 4 tench. However, 6 of the 7 virus positive fish were from stress tanks (Table 1). Although the prevalence of virus, in this trial, was lower than expected, particularly in the tench, the virus prevalence did appear to be higher in the tanks where the fish were exposed to stress. Furthermore, analysis of the water samples revealed elevated levels of cortisol release in the stress tanks compared to the non-stress tanks (Figs 1 & 2). This confirms that analysis of cortisol levels is a very efficient method of monitoring the effects of exposure to stress, especially brief acute exposures such as netting stress.

Table 1: Experimental study - Detection of virus in carrier fish after handling stress – Results summary

Tank	**09-40**	**09-39**	**09-38**	**09-37**	**09-36**	**09-34**	**09-33**	**09-32**
Species	Tench				Chub			
Stress	No	Yes	No	Yes	No	Yes	No	Yes
VHSV +ve fish	1/10	2/10	0/10	1/10	0/10	1/10	0/10	2/10
Virus titre (TCID50/ml)	$3.1x10^3$	1) $>10^5$ 2) PCR +ve		$9.9x10^4$		$5.6x10^4$		1) $9.9x10^3$ 2) $3.1x10^3$

Figure 1: Cortisol release rates in water containing stress-exposed and non-exposed chub.

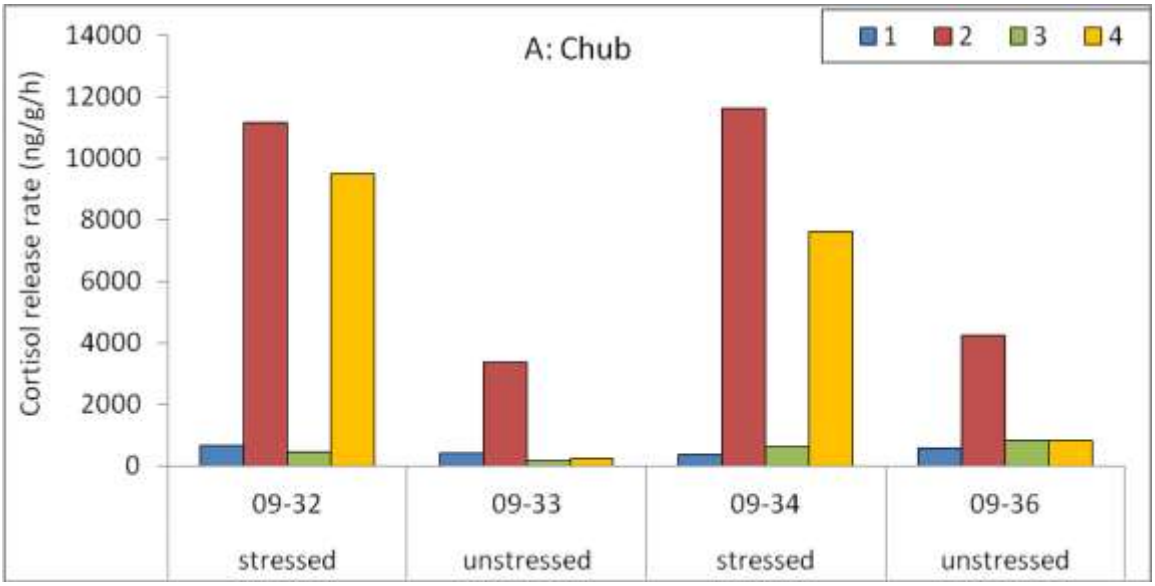
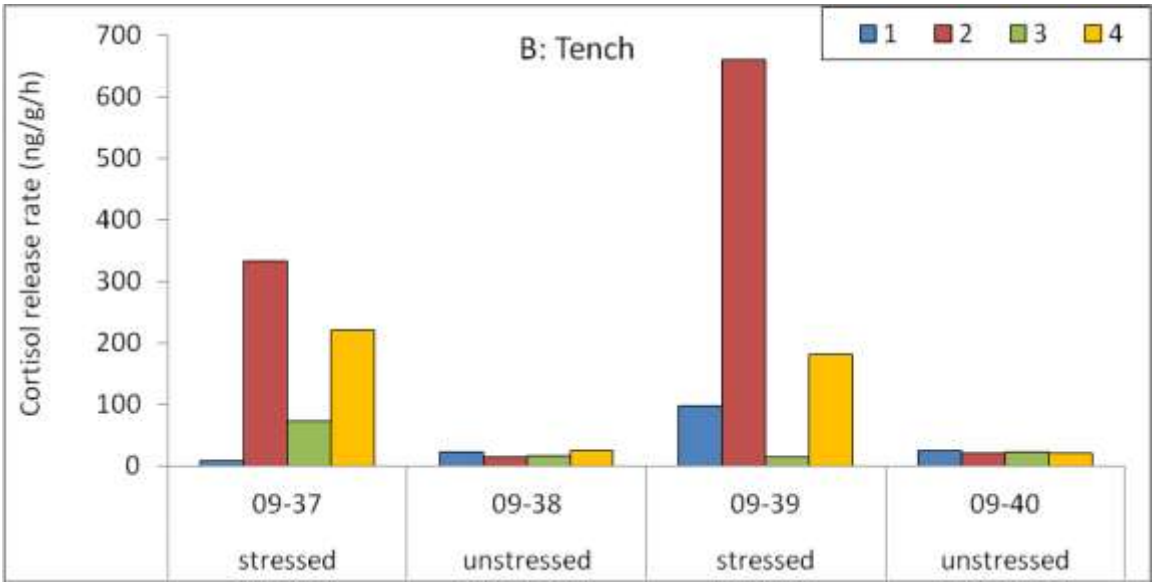


Figure 2: Cortisol release rates in water containing stress-exposed and non-exposed tench.



Footnote: The different coloured bars relate to the water samplings: 1 = 1st pre-stress sample (D14) ; 2 = 1st post-stress sample (D14) ; 3 = 2nd pre-stress sample (D21) ; 4 = 2nd post-stress sample (D21).

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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NOTE : There are no publications in the above list that are direct outputs of this project.