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Evidence Project Final Report

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Project identification

1. Defra Project code: F1199

2. Project title:
   Testing the susceptibility of Garra rufa to spring viraemia of carp (SVC) virus by ip injection.

3. Contractor organisation(s):
   Cefas Weymouth Laboratory
   Barrack Road
   The Nothe
   Weymouth
   Dorset, DT4 8UB

4. Total Defra project costs (agreed fixed price): £ 11,741

5. Project: start date 01-10-2011

   end date 30-04-2012

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

   Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

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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.

With the widespread importation of *G. rufa* into the UK for ichthyotherapy purposes there is a concern that the businesses using these fish (e.g. beauty parlours) have little or no experience in keeping fish and that there is a distinct possibility that unwanted live fish may be introduced into ponds, rivers and other natural waters. *G. rufa* are not known to be susceptible to SVCV, but being cyprinids, there is the possibility that they are susceptible. The full extent of sources of these *G. rufa* is not known but the potential exists, that if they are susceptible, they could be exposed to and become infected by the virus and introduce it into the UK. Hence to support risk based analysis decisions and the provision of advice regarding the introduction of SVC, the Fish Health Inspectorate would like to know whether *G. rufa* are susceptible to SVCV.

The proposed work was undertaken in two stages. The first stage was a preliminary challenge designed to provide evidence for susceptibility (>90% confidence) in individuals within the population at reasonably high prevalence (20%) by the invasive method of intra peritoneal injection (IP) but using only a few animals. Ten juvenile *G. rufa* were exposed to an infectious dose of up to $5x10^4$ $TCID_{50}$/fish of two different SVCV isolates of European and Asian origin by IP injection. 10 juvenile common carp were similarly injected as positive controls and a tank of each species was maintained as negative controls through sham injected with culture medium only.

In this preliminary challenge the viability of the virus isolates was confirmed since 60% of the positive control carp injected with European SVCV suffered mortality with clinical signs in all fish and high viral titres recovered from tissues. The Asian SVCV isolate did not cause mortality in carp but typical clinical signs were observed and viral titres recovered from fish sampled at 2 and 4 weeks post challenge were high indicating replication of virus. All negative controls were negative for SVCV.

In the *Garra rufa* challenged with SVCV (either isolate) there was no mortality observed. Virus was recovered from 40% (2 out of 5 fish) injected with European SVCV though clinical signs were not evident when sampled at 2 weeks and viral titres were relatively low ($3.78x10^2$ and $1.76x\ 10^3$ $TCID_{50}$/g visceral tissues). Clinical signs were observed in 20% (1 out of 5) fish injected with Asian SVCV sampled at 2 weeks and from which virus was recovered at low titre ($3.78x\ 10^2$). At 4 weeks post challenge virus was not detected in *G. rufa* injected with Asian SVCV but in 60% (3 out of 5) *G. rufa* injected with the European isolate, virus was still present (one at high titre – $2.58\ x10^5$) indicating persistence of the virus and limited evidence of viral replication for the European isolate.

This trial provided limited but insufficient evidence for susceptibility of *Garra rufa* to the SVCV isolates tested hence a second larger trail was undertaken using IP challenge again but designed to give sufficient statistical power to detect susceptibility at a much lower prevalence within the population (5% level with >90% confidence) rather than progress onto the more natural but less certain route of bath infection.

The second stage trial utilised duplicate tanks of 50 *G. rufa* with the same virus isolates but taking the source of the virus for preparation of stock culture from infected fish in the first trial. This passage in fish provided the potential to increase virulence of the isolates used.

Characteristic symptoms of SVC disease was induced in a number of *G. rufa* by IP injection of European isolate of SVCV and resulted in a combined cumulative mortality of 7% in two replicate tanks of 50 fish each, furthermore the dead and moribund fish contained a high titre of virus (up to 10^7 $TCID_{50}$/g of visceral tissue) indicating significant replication of virus. The mortality rate demonstrated in the *G. rufa* was substantially less than the carp controls.

Susceptibility of *G. rufa* to the Asian isolate tested was observed but markedly reduced with no mortality and very limited re-isolation of virus from sampled fish throughout the challenge period. This is most likely a reflection of the apparent reduced pathogenicity of isolates from this geographic area as indicated by the similar low mortality and viral replication seen in susceptible carp controls with this particular isolate.

Data obtained from fish sampled at 2 and 4 weeks post challenge supported the mortality data. Samples taken at 14 days post exposure and 28 days post exposure indicated a significant proportion of the fish (60%) retained the European SVCV virus within the first two weeks post challenge, and in 25% of these (3 fish) it was present at high titre indicating viral replication, but by 28 days the viral titre had dropped below detectable levels in the majority of remaining fish. Surprisingly no evidence of histopathological changes consistent with disease caused by viral infection was seen. This may be a reflection of the number of samples taken and the apparently low prevalence of susceptible individuals in the population.

It is apparent that there was no mortality and limited evidence for viral replication in the first stage trial and substantial evidence of viral replication and significant mortality in the second stage trial. A number of factors can be identified that may have contributed to these findings. The number of fish challenged between the two trials was significantly different and the small number used in the first stage may have precluded the opportunity to test susceptible fish within the population. Though purchased from the same retail outlet the batches of fish used in the two trials were different thus were likely to have been from different populations and hence potentially had different susceptibility. The virus used in the second challenge had been passaged recently through *G. rufa* and may have been more virulent for the second trial. The *G. rufa* used in the first trial were asymptomatic carriers of a serogroup C aquabirnavirus at low titre, whereas aquabirnavirus was not detected in the fish used in the second trial. It is possible the birnavirus infection had in some way activated the immune system in the fish retarding the development of SVCV infection and disease in the first trail. Further experimentation would be required to determine if these hypotheses were valid.

In conclusion *G. rufa* have been shown to be susceptible, at a relatively low prevalence within the populations tested, to disease and mortality caused by intra peritoneal injection of an isolate of SVCV of European origin.

Recommendations for future work.

There have been recommendations recently by organisations such as the OIE or EFSA that bath immersion or cohabitation, because it is non invasive, are challenge methods preferred for demonstration of susceptibility of various host species. It is recognised that bath exposure is often likely to be a less efficient means of infection for the first approach to testing disease susceptibility; hence based on ethical grounds of reduced animal usage, IP injection was used in the first instance for the disease challenges in this project. Had the evidence for susceptibility been stronger in the first stage trial, then the challenge method used in the second trial would have been bath immersion. It is now appropriate to undertake a challenge with a sufficient number of *G. rufa* to detect the lower prevalence of susceptibility but using the more natural route of infection preferred by OIE and EFSA for demonstration of susceptibility, recognising however that the mortality and disease observed may be reduced.

It may then be informative to investigate the potential for *Garra rufa* to develop a carrier status for SVCV. This could be done by bath exposing *G. rufa* then transferring fish at intervals of 2, 4 and 6 weeks post exposure to new tanks containing susceptible naive carp for cohabitation.

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).

Objective

To determine if Garra rufa are susceptible to infection, support replication of the virus and/or succumb to disease caused by representative isolates of European and Asian SVCV after intra-peritoneal injection and or bath emersion.

The proposed work was undertaken in two stages.

Stage 1:

The first stage was a preliminary challenge designed to provide evidence with >90% confidence for susceptibility in individuals within the population if at a reasonably high prevalence of 20% by the invasive method of intra peritoneal injection (IP) but using only a few animals (10 per species per isolate).

Materials and Methods:

Fish - *Garra rufa* (av. weight 1.3g and av. standard length 4.5cm) were purchased from a local aquatic trade shop and carp (av. weight 5.2g and av. standard length 5.0cm) were purchased from a local breeder.

PCR amplification and sequencing of a partial fragment of the cytochrome b gene was used to confirm the species identity of the fish as *Garra rufa.*

A sample of 10 fish of both species was tested for initial health status by bacterial and viral isolation on media and cell culture respectively. For virus isolation visceral homogenates were inoculated onto EPC and FHM cell lines an incubated for 14 days at 20°C and 25°C with passage after 7days. A specific RT-PCR test was performed on homogenates and cell culture supernatant for detection of SVCV.

Fish sampled for health check showed no external or internal gross clinical signs and there were no significant bacterial counts for all fish from both species. SVCV was not detected either by cell culture or RT-PCR. Cytopathic effect (CPE) was seen in FHM cells inoculated with *G. rufa* visceral homogenate at 10^{-2} dilution and incubated at both 20 and 25°C. the cell culture supernatant was tested by RT-PCR with nested generic birnavirus primers. A product of 226bp was amplified which when sequenced showed 99% identity to serogroup C aquabirnaviruses indicating the *G.* rufa harboured a low level birnavirus infection in 50% of the sample. A previous batch of *G. rufa* purchased for these experiments had also carried the serogroup C birnavirus and a significant number (3 out of 9) of previous G. rufa samples taken for import checks by the Cefas Fish Health Inspectorate and tested in the Diagnostic Virology Laboratory have also been shown to carry a serogroup C aquabirnavirus. It became apparent therefore that it could be difficult to obtain *G. rufa* that were free of this aquabirnavirus and the search for these fish could be costly both in time and finances purchasing fish. The decision was made to use these birnavirus carrying *G. rufa* in the challenges with SVCV.

The SVCV virus isolates used were European SVCV isolate 940500 at passage 3 and Asian SVCV isolate H243.3 at passage 1 both grown on EPC cells at 20°C. The titres of the stock virus used were 2.58×10^5 and 1.2×10^6 $TCID_{50}$/ml for European and Asian isolates respectively.

10 juvenile *G. rufa* were exposed to an infectious dose of 1.29×10^4 or 6×10^4 $TCID_{50}$ per fish for European and Asian SVCV isolates respectively by IP injection of 50µl of virus culture medium. 10

juvenile common carp were similarly injected as positive controls and a tank of each species sham injected with culture medium only was maintained as negative controls. Fish were held at 15°C for 28 days and 5 fish were sampled at 14 and 28 days post exposure. Samples were processed for virus and bacterial isolation. Visceral tissue homogenates were inoculated onto BF and EPC cells. The head containing a proportion of the head kidney was fixed in formalin for analysis by histopathology.

Results:

The viability of the virus isolates was confirmed since 60% of the positive control carp injected with European SVCV suffered mortality with clinical signs in all fish and high viral titres were recovered from tissues (up to $2.58x10^7$ $TCID_{50}$/g visceral tissues). The Asian SVCV isolate did not cause mortality in carp but typical clinical signs were observed and viral titres recovered from fish sampled at 2 and 4 weeks post challenge were high (up to 3.78×10^6 $TCID_{50}$/g fish) indicating replication of virus. All negative controls were negative for SVCV.

In the *Garra rufa* challenged with SVCV (either isolate) there was no mortality observed. Virus was recovered from 40% (2 out of 5 fish) injected with European SVCV though clinical signs were not evident when sampled at 2 weeks and viral titres were relatively low ($3.78x10^2$ and $1.76x\ 10^3$ $TCID_{50}$/g visceral tissues). Clinical signs were observed in 20% (1 out of 5) fish injected with Asian SVCV sampled at 2 weeks and from which virus was recovered at low titre ($3.78x\ 10^2$).
At 4 weeks post challenge virus was not detected in *G. rufa* injected with Asian SVCV but in 60% (3 out of 5) *G. rufa* injected with the European isolate, virus was still present (one at high titre – $2.58\ x10^5$) indicating persistence of the virus in a number of fish and limited evidence of viral replication for the European isolate (a single fish). A proportion of the viral isolations from challenged fish were confirmed as SVCV and of the appropriate isolate by specific RT-PCR amplification and sequencing. Histopathology indicated potential changes associated with viral disease in the head kidney of a single *G. rufa* these included large renal macrophage aggregates and mild haemopoetic cell disruption or necrosis.

Preliminary summary
From this we concluded there was some but limited evidence for susceptibility of *Garra rufa* to the SVCV isolates tested. Had the evidence for susceptibility been stronger in this preliminary challenge the second stage would have been to confirm susceptibility by a more natural route of infection, namely challenge by bath exposure. Based on the limited evidence however we decided a larger IP challenge was more appropriate.

Stage 2:
The second stage was a repeat injection challenge using the same virus isolates and duplicate tanks of 50 *G. rufa*. These numbers give sufficient statistical power to detect susceptibility at a much lower prevalence within the population (5% level with >90% confidence). There is evidence that passage of viruses in cell culture can attenuate virulence and that passage through live hosts can increase pathogenicity of viruses. Thus to potentially enhance or maintain virulence of the isolates and to provide the greatest opportunity for disease progression in the second stage trial the same virus isolates were used but the source of virus used to prepare stock was derived from infected fish tissues from the first trial.

Materials and Methods:
The carp used in the second trial were from the same population as those used in the first trial. The *G. rufa* used were a separate batch of fish purchased from the same aquatic trade outlet. The *G. rufa* were slightly smaller (av. weight 1.0g and av. standard length 3.7cm) and the 10 fish sample health check indicated the fish in this batch were not infected with aquabirnavirus.

Amplification and sequencing of a partial fragment of the cytochrome b gene again confirmed the fish were of the *Garra rufa* species.

The virus stock prepared from tissues of *G. rufa* infected with the SVCV isolates in the first challenge was grown in EPC cells which do not support the growth of aquabirnavirus and the SVCV stock prepared from the challenged *G. rufa* in this manner was tested for presence of aquabirnavirus by passage onto BF cells (permissive for aquabirnavirus growth) and by specific RT-PCR. Stock SVCV virus for the second trial was shown to be free from co-infection with aquabirnavirus.

Duplicate tanks of 50 juvenile *G. rufa* were exposed to an infectious dose of $1x10^4$ $TCID_{50}$ per fish for both SVCV isolates by IP injection of 50µl of virus culture medium. 10 juvenile common carp were

similarly injected as positive controls and a tank of each species sham injected with culture medium only was maintained as negative controls. Fish were monitored for 28 days and 10 fish were sampled for virology and 10 fish sampled for histopathology from each tank at 14 and at 28 days post exposure. Samples were processed for virus and bacterial isolation. Visceral tissue homogenates were inoculated onto EPC cells. Whole *G. rufa* were fixed in formalin for analysis by histopathology.

Results:

Virulence of the isolates was re-confirmed as clinical signs of disease and mortality of 80% (8 out of 10) fish injected with European SVCV was observed in the positive control carp (Figure 1A) with viral titres recovered from dead fish ranging from $5.56x10^3$ to $1.2x10^7$ $TCID_{50}$/g visceral tissue. Mortality was also seen in carp injected with Asian SVCV (Figure 1 A) where 2 out of 10 fish (20%) succumbed to the virus with recovered viral titres of $1.2x10^5$ and $2.58x10^7$ $TCID_{50}$/g of visceral tissue.

No mortality was observed in negative control tanks and all fish were negative for viral isolation at the end of 28 days challenge.

In *Garra rufa* in contrast to the first trial, mortality was observed in fish injected with European SVCV (Figure 1B). Cumulative mortality reached 4% and 10% (2 and 5 out of 50 fish respectively) in the duplicate tanks after 28 days. Mortality commenced at 10 and 8 days for the two tanks respectively. Clinical signs were observed including impaired swimming ability, distended abdomen and ascetic fluid in the peritoneal cavity. Viral titres recovered from the mortalities were all significantly higher than the challenge inoculum and ranged from $8.15x10^5$ to $5.56x10^7$ $TCID_{50}$/g of visceral tissue (Table 1). There was a single mortality in one of the duplicate tanks at one day post exposure, which was attributed to injection damage and was omitted from the mortality curve data, virus was recovered from this individual at a titre of $1.76x10^3$ $TCID_{50}$/g of visceral tissue which is similar to but lower than the challenge inoculum.

In *G. rufa* injected with the Asian SVCV isolate there were an initial 4 mortalities (2 from each duplicate tank) at one day post injection which were attributed to injection damage and not included in the mortality curve but there was no subsequent mortality for the remainder of the 28 day challenge (Figure 1B).

In sampled fish, virus was recovered from 12 of the 20 *G. rufa* (60%) injected with European SVCV when sampled at 14 days post exposure. Viral titres ranged from $2.58x10^2$ to $1.2x10^7$ $TCID_{50}$/g of visceral tissue (Table 2). The recovered viral titres in three individuals were significantly higher than the challenge inoculum, a clear demonstration of replication of the virus within the fish and indication that the individuals would likely have died subsequently. The individual which produced the highest viral titre also showed petechial haemorrhaging of the eyes and ventral skin surface (Figure 2).

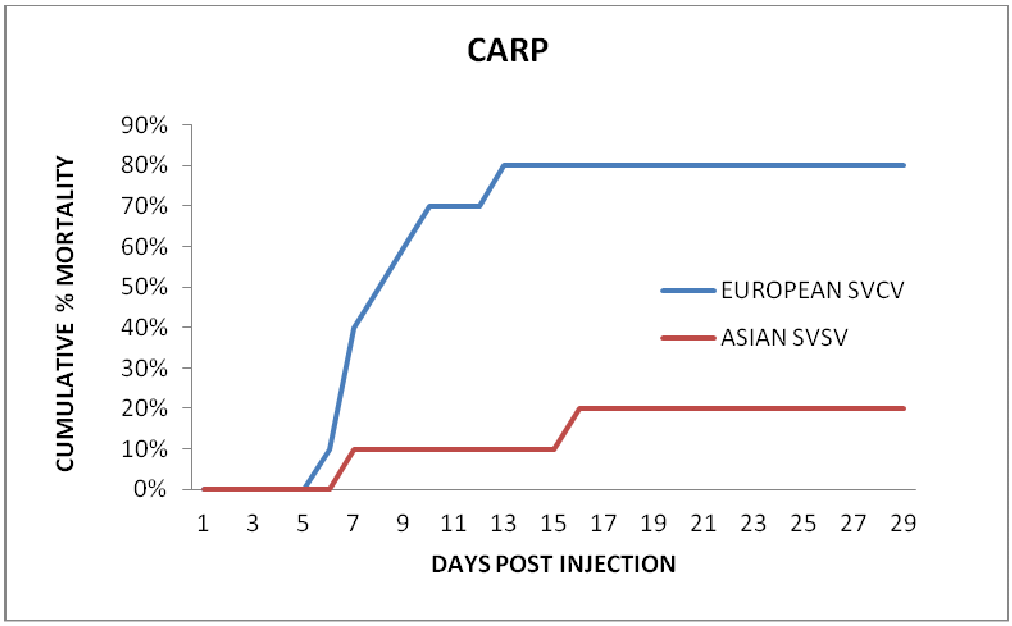
In contrast virus was recovered from only 2 of the 20 sampled *G. rufa* (10%) injected with Asian SVCV when sampled at 14 days post exposure and viral titres recovered were $3.78x10^4$ and $2.58x10^2$ $TCID_{50}$/g of visceral tissue (Table 2).

There was a marked decrease in viral recovery from fish sampled at 28 days post exposure. Virus was recovered from 3 of the 20 sampled *G. rufa* (15%) injected with European SVCV and 1 of the 20 sampled *G. rufa* (5%) injected with Asian SVCV. Viral titres were low at between 2.58 and 8.15 $x10^2$ $TCID_{50}$/g of visceral tissue.

A representative sample of virus isolations from G. rufa were subjected to RT-PCR and sequencing for confirmation of the virus genogroup re-isolated.

10 fish from each tank were sampled at each of the two time points for analysis of histopathology. *G. rufa* were fixed and sectioned whole. Surprisingly analysis did not show significant histopathological changes consistent with viral infection in the sampled fish. This may be a reflection of the sample size and the low prevalence of susceptible fish within the population.

A



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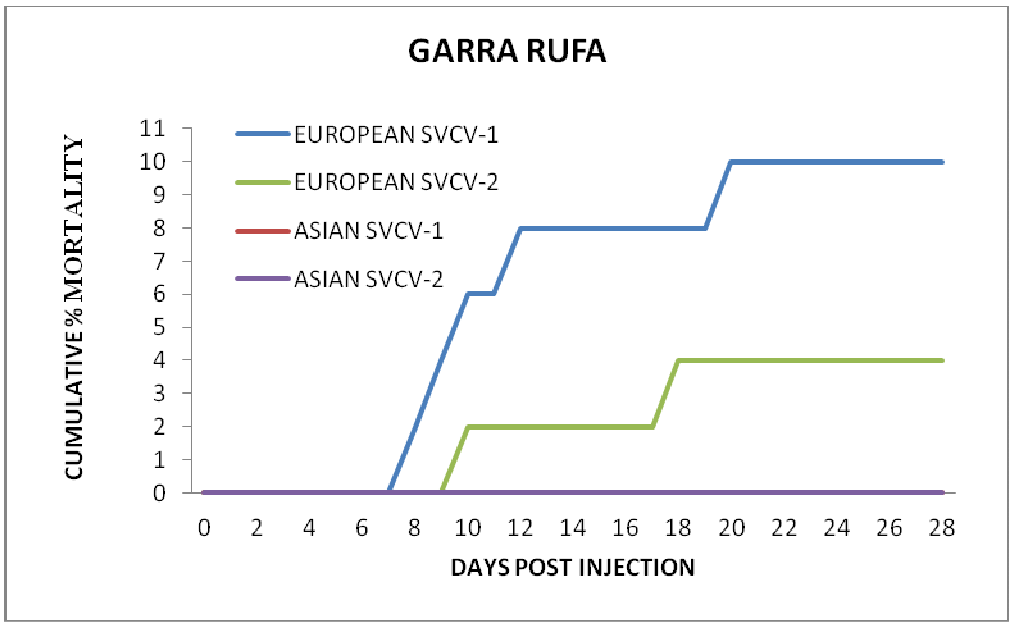

Figure 1. Cumulative percent mortality curves for carp and Garra rufa injected with SVCV isolates of Asian and European origin. Single tanks of 10 carp and duplicate tanks of 50 *G. ruffa* were challenged for each isolate. Negative controls sham injected with control medium only showed no mortality (data not shown).

Table 1. Mortality data and recovered viral titre ($TCID_{50}$/g of visceral tissue) for *Garra rufa* injected with European SVCV

Mortality sample no.	Days post injection	Titre in EPC cells
Tank 11-29		
1	1	1.76×10^3
2	8	5.56×10^7
3	9	1.76×10^7
4	10	3.78×10^7
5	12	8.15×10^6
6	20	1.2×10^7
Tank 11-30		
1	10	8.15×10^6
2	18	8.15×10^5

Table 2. Virus titres recovered from sampled Garra rufa 14 days post exposure to European and Asian isolates of SVCV virus.

European SVCV		Asian SVCV	
Sample	**Titre in EPC cells**	**Sample**	**Titre in EPC cells**
Tank 11-29		**Tank 11-31**	
1	2.58×10^2	1	-
2	2.58×10^2	2	-
3	-	3	-
4	-	4	3.78×10^4
5	-	5	2.58×10^2
6	3.78×10^3	6	-
7	5.56×10^4	7	-
8	3.78×10^3	8	-
9	-	9	-
10	-	10	-
Tank 11-30		**Tank 11-32**	
1	-	1	-
2	2.58×10^2	2	-
3	-	3	-
4	3.78×10^2	4	-
5	1.2×10^7 a	5	-
6	1.2×10^6	6	-
7	2.58×10^3	7	-
8	-	8	-
9	5.56×10^2	9	-
10	2.58×10^5	10	-

a – petechial haemorrhages observed in eyes and on ventral skin surface.

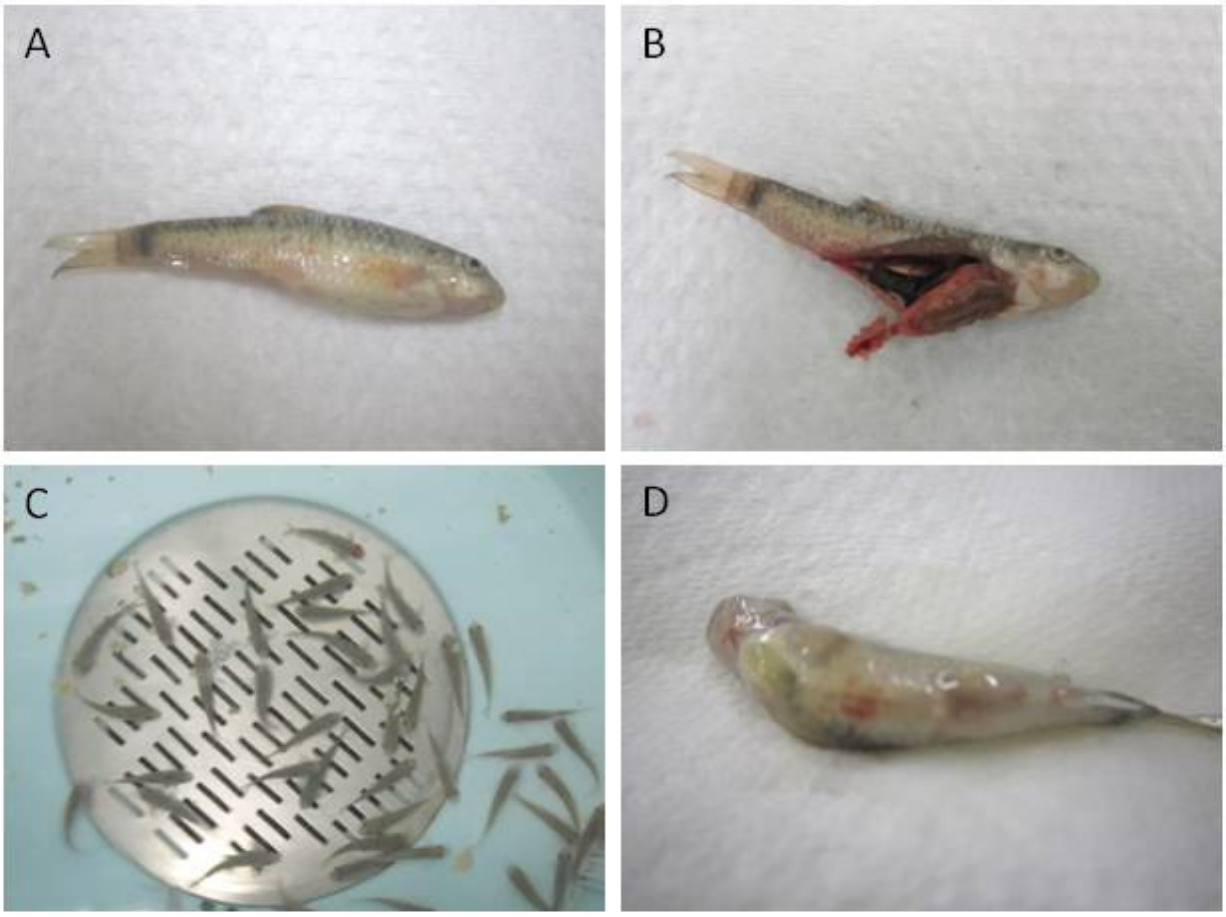

Figure 2. *Garra rufa* infected with SVCV. *Garra rufa* sampled in the stage 1 challenge showing petechial haemorrhages of the skin in the abdominal area (A) and internal haemorrhages (B). Second stage challenge tank setup (C). *Garra rufa* mortality 9 days after of i.p. injection with the European SVCV isolate, showing distended abdomen and external haemorrhages (D).

Summary:

This second trial provides evidence for low to moderate susceptibility of *Garra rufa* to the European isolate of SVCV when administered by intraperitoneal injection. Characteristic symptoms of SVC disease was induced in a number of *G. rufa* and resulted in a combined cumulative mortality of 7% in two replicate tanks of 50 fish each, furthermore the dead and moribund fish contained a high titre of virus (up to 10^7 $TCID_{50}$/g of visceral tissue) indicating significant replication of virus. The susceptibility demonstrated in the *G. rufa* was substantially less than the carp controls. Susceptibility of *G. rufa* to the Asian isolate tested was markedly reduced with no mortality observed and very limited re-isolation of virus from sampled fish throughout the challenge period. This is most likely a reflection of the apparent reduced pathogenicity of isolates from this geographic area as indicated by the similar low mortality seen in susceptible carp controls with this particular isolate. Samples taken at 14 days post exposure and 28 days post exposure indicated a significant proportion of the fish (60%) retained the European SVCV virus within the first two weeks post challenge, and in 25% of these it was present at high titre indicating viral replication, and though by 28 days the viral titre had dropped below detectable levels in the majority of remaining fish virus remained in some individuals.

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.

Confirmation of susceptibility by bath exposure or cohabitation is likely to be required before this work could be published in a peer reviewed journal.