

Case report

Outbreak of Mortality Associated with Acipenser Iridovirus European (AcIV-E) Detection in Siberian Sturgeon (*Acipenser Baerii*) Farmed in Sweden

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Abstract

Background: Infectious disease is a major challenge in aquaculture and poses a constraint for development of farming of new species. In 2017, Siberian sturgeon (*Acipenser baerii*) juveniles were imported from Italy to a Swedish farm. Due to stressful conditions, 30% died during transport and in the first days after arrival. Ten days after arrival, mortalities started to occur again. Within two months, only 5% of the juveniles were still alive.

Methods: Diseased fish were transported live to the National Veterinary Institute (SVA) for necropsy and further analysis. Pathological and histopathological investigation was conducted. Virology was performed on gills and internal organs by cell culture isolation and specific PCR protocols.

Results: The juveniles displayed neurological signs such as lethargy, inability to maintain upright position and erratic swimming. Body condition was low. Gills were pale. One fish had petechial hemorrhage on the abdomen and the snout. The ventricles were air-filled with, but swim bladders were deflated. One specimen had intestinal hemorrhage. Viral cell cultures were negative, but PCR of gills and internal organs detected the presence of Acipenser Iridovirus European (AcIV-E).

Conclusions: AcIV-E was associated with disease and high mortality in the sturgeon juveniles. Stress probably aggravated the course of the infection.

Keywords: Siberian sturgeon; CNS signs; Acipenser iridovirus European; viral nervous necrosis; Acipenser herpesvirus; gas bubble disease; Polymerase chain reaction

1. Introduction

Sturgeon farming has been increasing in the last decade due to the desire for caviar and for sturgeon meat [1, 2]. Despite the fact that sturgeon is a rough species and have adapted well to farming conditions, infectious disease is one of the major challenges in sturgeon aquaculture due to limited knowledge about epidemiology and control methods [1]. In addition, none of the main viral infections (Nervous necrosis virus (NNV), Acipenser iridoviruses, Acipenser herpesviruses and Acipenser adenovirus) that can cause extensive losses in sturgeon farms are regulated in the OIE or EU legislations. This means regular screening for viral disease and restrictions for trade is poorly implemented, contributing to spread of pathogens through sub clinically infected individuals.

In recent years several mortality events in different sturgeon farms across Europe have been investigated [1, 3]. The same etiological agent, a new virus, was detected and associated with clinical

disease in different sturgeon species (*A. baerii*, *A. gueldenstaedtii*, *A. naccari*, *Huso huso*). The virus - Acipenser iridovirus European (AcIV-E) - appeared to be closely related with North American sturgeon iridoviruses, in particular with the white sturgeon iridovirus (WSIV) and the Namao virus (NV). The taxonomical classification of these viruses is yet to be elucidated. They have recently been moved from *Iridoviridae* to *Mimiviridae* [4, 5], a viral family characterized by extreme genomic complexity and plasticity. As a matter of fact, the genomes of these viruses are still largely unknown. Additionally, much other important information, e.g. pathogenesis, transmission routes and epidemiology of these viruses is lacking, making virus and disease control extremely difficult. Unfortunately, all virus isolation attempts performed have failed, so the way to comprehension of many of these aspects appear long and complicated.

In the summer of 2017, a new-built recirculating aquaculture system (RAS) farm in Sweden received a batch of 2000 Siberian sturgeon (*Acipenser baerii*) juveniles from an Italian farm. Due to disease and mortalities a diagnostic investigation was performed.

2. Results

2.1. Case history

The Swedish farm had ordered 7 g juveniles, but at arrival the average weight was 3,5 g. The age of the juveniles was not stated, but according to a growth curve it takes approximately 45 days for Siberian sturgeon juveniles to reach 3.5-4 g (the Swedish farmer, personal communication). This was the first fish introduced to the farm, thus no disease could have been present at the site prior to this import. The juveniles were transported in aerated plastic bags inside a box. The box had not been sent with live animal markings, and it is unclear whether it was placed in a pressure cabin or not during the flight. In addition, delivery at the border control was delayed with several hours since the package was not sent to the animal border control station. When reaching the farm, the fish had been transported for 12-14 hrs. All juveniles were put together in a 20 m³ tank, producing a fish density of 350 g/m³. Water temperature was approx. 20°C. More tanks without fish were run in parallel to start up the facility. The water was mainly recirculated, but approximately 15 m³ of municipal water that had settled in sedimentation tanks for 36-48 hrs before being transferred to the fish tanks was added each day. Water quality was continuously monitored. During transport and in the next few days, 30% of the juveniles died. The remaining 70% of the batch seemed to settle properly, but about 10 days after arrival, the juveniles started to display buoyancy problems, lethargy and anorexia and mortalities started to occur again. The day before that, the farmer had altered the water inlet by mixing well water into the municipal water. No surge in dissolved nitrogen, oxygen or other water parameters had been recorded but the farmer suspected gas bubble disease and returned to a full municipal water supply. Still, disease and mortalities continued. After another week, a diagnostic investigation was started to look deeper into the problem and rule out infectious disease.

2.2. Clinical observation and necropsy

In the first batch of fish sent for diagnosis (in July, n=6), all specimens displayed varying degrees of central nervous system (CNS) signs with erratic swimming, buoyancy and equilibrium problems or complete lethargy (**Video S1**). Most fish hyperventilated, and all fish had a body condition below average (**Figure 1a**). At necropsy, eyes were clear and there was no sign of exophthalmia or presence of gas bubbles in the skin or mouth. No external parasites were identified at direct microscopy of skin and gill scrapings. Gills were pale, and abdomens distended. One fish had petechial hemorrhage of the skin at the pelvic fins and the anus (**Figure 1b**). The abdominal distension was caused by air-filled ventricles (**Figure 1c**). No or scarce feed residues were present in the gastrointestinal system. Swim bladders were completely deflated in all fish (**Figure 1d**). The intestine was hyperemic in two fish (**Figure 1d**). Spleens could not be identified. Kidneys were thin and pale.

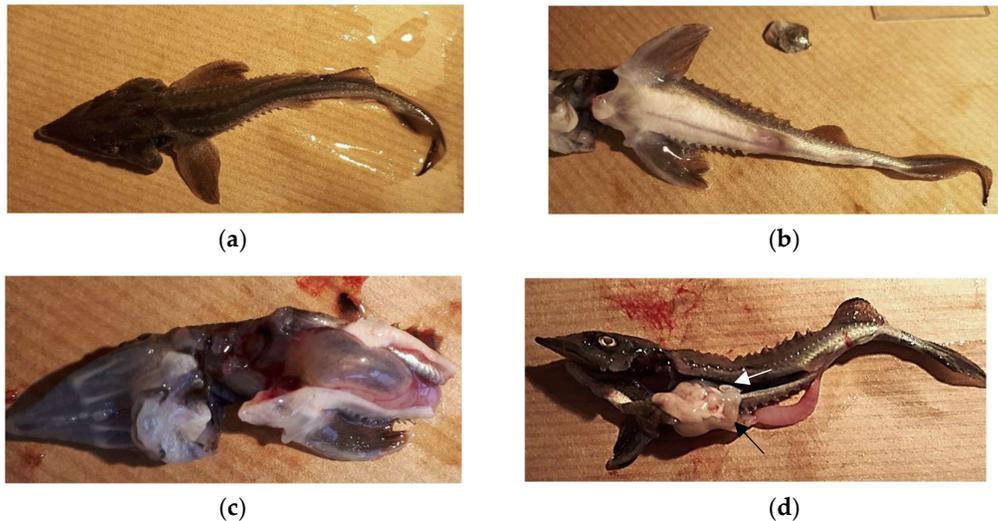


Figure 1. Symptoms at necropsy of the first six sturgeon juveniles sent in July. (a) Low body condition (b) Petechial hemorrhage in the pelvic fin area (c) Severe distension of the ventricle (d) A deflated swim bladder (white arrow), a ventricle not distended by air (black arrow) and a fluid filled, hyperemic intestine.

From the second batch of five diseased and five “healthy” juveniles (August, n= 10), six displayed the same CNS signs as described above and were thin. Gills were slightly to moderately pale. In a few fish, pupils were dilated, and the skin was darker than normal. Hematocrit was measured and was apparently lower in the diseased group (21-30% (n=3) than in the “healthy group”, (29% (n=1), 45-53% (n=4)), but non-significant by Fisher’s exact test. For two juveniles in the diseased group, hemolysis made interpretation of hematocrit impossible.

2.2. Histology and cytology

Severe epithelial hypertrophy and mild lymphocytic infiltration was seen in the gill epithelium, but typical iridoviral inclusions were not seen (**Figure 2a**). Necrotic lesions or presence of vacuoles indicative of viral nervous necrosis (VNN) could not be seen in the brain. One potential iridovirus inclusion was seen in the lip of one fish (**Figure 2b**). Gas bubbles could not be detected in any of the investigated tissues. Blood smears showed regenerative anemia, with a high number of degenerated erythrocytes, erythroblasts and proerythrocytes (**Figure 3**). Anemia and regeneration were more prominent in July than in August.

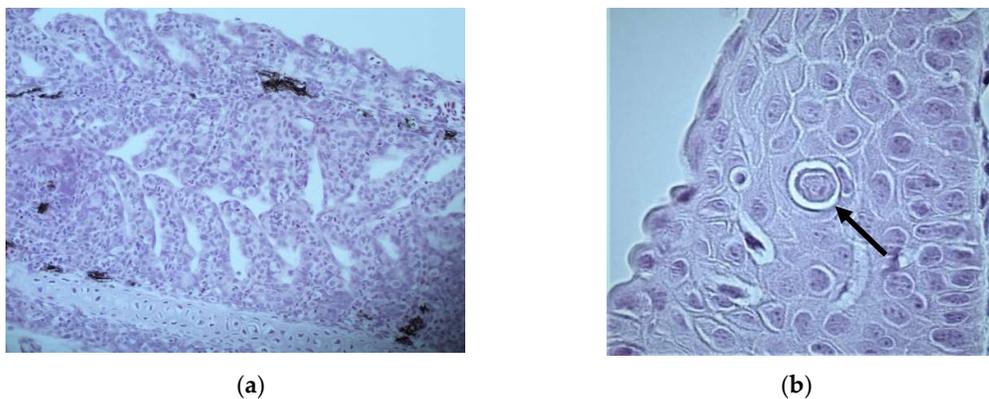


Figure 2. (a) secondary gill lamellae with severe epithelial hypertrophy (b) close up of lip epithelium with a potential iridovirus infected cell (black arrow).

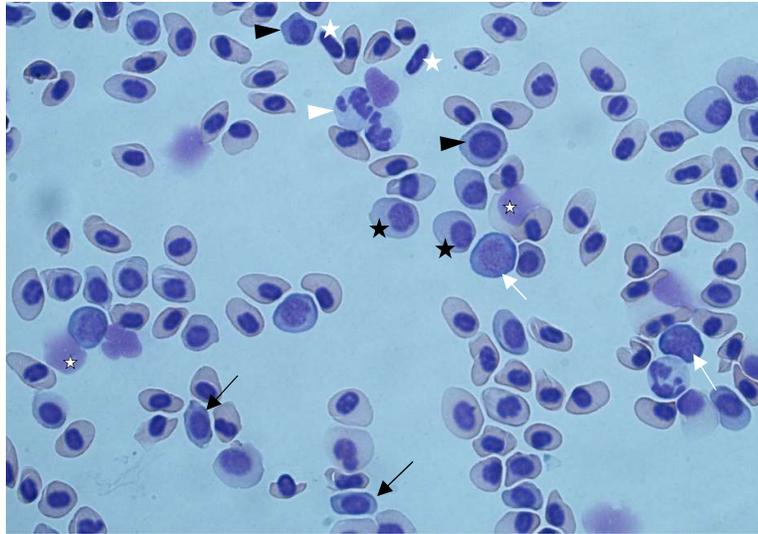


Figure 3. Blood smear (x100) from one of the juveniles sampled in July. Mature erythrocytes are deformed, sometimes with fragmented nuclei. Erythroblasts (black arrowhead), proerythrocytes (black arrow), swelling erythrocytes (black star) and a few smudge cells (white star, black outline) are present. Thrombocytes (white star), neutrophils (white arrowhead) and monocytes (white arrow) are also present in the photo.

2.3 Virology and bacteriology

All samples used to inoculate cell cultures were negative despite the multiplicity of cell lines and temperature tested (**Table 2**, Materials and Methods section). Qualitative real time Polymerase Chain Reaction (qPCR) of internal organs (including the brain) from two individuals in July were negative for NNV but gave faint signals for AcIV-E. From one of the samples, an amplicon could be obtained by conventional PCR. The amplicon was sequenced, confirming presence of AcIV-E (**Table 1**, sample 1-1). Subsequent qPCR of gill tissue, considered to be the target tissue for AcIV-E detection [2], collected in August provided further evidence for the presence of AcIV-E DNA (**Table 1**, samples 2-1 – 2-9). Conventional PCR confirmed qPCR results and a partial sequence of the MCP gene (1303 bp) was obtained. Sequence analysis revealed a nucleotide similarity of 99% with already published sequences of AcIV-E. The new sequence was submitted to GenBank under the accession number MH817858.

No bacterial growth except for a sparse mixed flora in the ventricle of one fish was detected.

Table 1. Results from qPCR, conventional PCR and sequencing of the MCP gene of AcIV-E in nine sturgeon samples

Batch - fish	Status of individual	organ	qPCR (CT) ¹	Conventional PCR	Sequencing
1-1	diseased	Internal (pool)	Doubt (37.57)	Pos	AcIV-E-
1-2	diseased	Internal (pool)	Doubt (38.06)	Neg	ND
2-1	diseased	Gills	Neg (no CT)	Neg	ND
2-2	diseased	Gills	Doubt (39.38)	Neg	ND
2-3	diseased	Gills	Pos (29.78)	Pos	AcIV-E
2-4	diseased	Gills	Neg (no CT)	Neg	ND
2-5	diseased	Gills	Doubt (37.48)	Neg	ND
2-7	“healthy” with clinical signs	Gills	Pos (32.47)	Pos	AcIV-E

2-9	“healthy”	Gills	Neg	Neg	ND
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¹ Threshold value (CT) for positive samples = 37.00 cycles; Doubtful = 37.00-39.00 cycles; Negative > 39.00
ND = not done

3. Discussion

The only pathogenic agent found in diseased sturgeon was AcIV-E. AcIV-E has been described in both Siberian and Russian sturgeon [1,3]. In an Italian farm farming both species, higher mortality due to AcIV-E was present in Russian sturgeon compared to Siberian sturgeon - 90% vs 50% [1] - suggesting a lower susceptibility to the virus in Siberian sturgeon [1, 3]. In the case described here, a cumulative mortality of 95% (including the initial 30% mortality around transport) was reached in two months from arrival in the imported batch of juvenile Siberian sturgeon.

Acipenser iridoviruses infect epithelial cells in gills, oropharynx, nasal organs and the epidermis [6] and are known to cause a chronic wasting syndrome [7]. However, infection has also been associated with neurological signs [1,2]. The farmers suspicion of gas bubble disease was not considered a probable diagnosis after observation of live juveniles and necropsy, since the neurological signs and necropsy findings together strongly indicated presence of viral infection based on previous observations [1, 2, 7]. Clinical signs agreed with what has been described for AcIV-E in Russian sturgeon [2] but infection with NNV was also a possibility. Histopathology was not indicative of NNV infection. Gill epithelial hypertrophy could indicate iridoviral infection, but only one potential iridoviral inclusion body was found in the lip epithelium, and molecular biology analyses confirmed presence of AcIV-E and absence of NNV in the samples tested. Low prevalence of typical iridoviral histological lesions in AcIV-E infected Siberian sturgeon has been already described [3] and thus the histopathology in this case is in line with previous findings.

Regenerative anemia can be an effect of viral infection. In line with the epitheliotropic nature of sturgeon iridoviruses, it should not be present, and it has not been described in any of the available references regarding AcIV-E. However, anemia is common in infections with systemic iridoviruses such as Red seabream iridovirus (RSIV) and Infectious spleen and kidney necrosis virus (ISKNV) [8] in fish. Further investigations of new cases are needed to confirm or discard this clinical sign as caused by AcIV-E. The observed hyperventilation was probably due to both anemia and the infection/hypertrophy of the gill epithelium – both causing a decreased ability to extract oxygen from the water, inducing hypoxia. Hypoxia should also be the cause of distended ventricles, if fish gasped for air at the surface and thereby swallowed it. Air-filled ventricles and intestines have also been observed in other AcIV-E outbreaks (A. Toffan personal communication).

There were no indications of concurrent bacterial disease affecting the fish, but bad transport conditions probably contributed to the high mortality by inducing severe stress, predisposing infected individuals to develop disease and increasing infection susceptibility in uninfected individuals [9], thus triggering onset of clinical disease from a latent infection.

Infectious disease is a major bottleneck for the development of aquaculture activities and especially viral disease represent a challenge due to the lack of treatment. Disease control in sturgeon farming is difficult due to lack of knowledge about disease epidemiology and control methods [1]. Thus, there is poor implementation of preventive and control measures for viral disease of sturgeon. Further problems arise since none of the sturgeon viruses are controlled through legislation. EU member state (MS) authorities only take EU regulated diseases and those diseases that the MS have additional guarantees for into account at import. For Sweden, that has free status for all EU listed fish diseases, and no other serious viral diseases being detected regularly, this poses a major threat to fish health. This case is one example of what can happen when potential serious infections are overlooked when fish is transferred from one farm to another, whether crossing a border or not. For aquaculture in general, not only for Sweden, an overview of diseases, and in particular the viral ones, in other species than salmonid fish is necessary. For example, the provision of species or fish group specific recommendations for health certificates at sale both regionally and internationally could be suggested

to prevent the spread of diseases with large economic losses for the purchasing farms, as well to prevent introduction of serious disease to new regions. This would also provide a knowledge basis and a leverage for MS authorities to apply for additional guarantees regarding specific diseases threatening important aquaculture species in their countries.

4. Materials and Methods

Juveniles were sent live in two batches with six (all diseased, July) and ten (five diseased, five considered as healthy by the farmer, August) fish each to SVA for necropsy and further analysis. Euthanization was performed using buffered tricaine (MS-222) by adding 5 ml of 25 mg/ml MS-222 solution and 4,5 ml of 20 mg/l NaHCO₃ buffer in a bucket containing 1 L of water and sturgeon juveniles. For the second batch, two buckets were used to keep the two groups separate. Fish were immediately necropsied, and tissues were sampled from fish with apparent clinical signs and from the second batch also from two fish without symptoms (of which samples from one fish were used) for various analyses as described below.

Hematocrit was measured in the second batch of fish. Blood smears were made by cutting the tail and one drop of blood was put on a glass slide and spread out using another slide. Dried blood smears were stained with May Grünwald Giemsa by a modified protocol from Pappenheim, 2012 [10]. Selected tissues (brain, kidney, liver, gills, spleen, ventricle) and two whole juveniles were preserved for histology in 4% buffered formaldehyde for 5 days. Tissues were then embedded in paraffin and prepared for microscopy by standard procedures. Sections were stained with Haematoxylin-Eosin (HE). Sections were read at 2-100 X using light microscopy.

Other fish than salmonids are still rare in Swedish aquaculture, and viral diagnostics for non-salmonid viruses except SVC and KHV are not present at SVA. From the first batch, internal organs (kidney, heart, spleen and brain) were thus put in Eagles minimum essential medium (EMEM), prepared for cell culture inoculation, frozen at -70°C as two subsamples and sent on dry ice to the EU reference laboratory for fish in Kgs. Lyngby, Denmark for viral cultivation and to Istituto Zooprofilattico Sperimentale delle Venezie, Italy for PCR and subsequent sequencing. From the second batch of fish, individual organs (kidney, heart, spleen, brain and gills) were also put in PCR tubes and frozen at -70°C. Gill samples from all five diseased and two “healthy” (of which one had clinical signs of disease) were sent on dry ice to Istituto Zooprofilattico Sperimentale delle Venezie, Italy for PCR and subsequent sequencing. Viral cultivation was performed on pooled samples of spleen, kidney, heart and brain. Samples were grinded with sterile sand quartz in sterile cooled mortars, diluted 1:10 in EMEM supplemented with 10% fetal bovine serum, 2% of antibiotic/antimycotic solution–10.000 IU/ml penicillin G, 10 mg/ml streptomycin sulfate, 25 µg/ml amphotericin B–0.4% of 50 mg/ml kanamycin solution) and centrifuged at 4000 g at 4°C for 15 min. Supernatants were collected and kept at 4°C overnight to give the antibiotics time to inhibit bacterial growth. Thereafter supernatants were inoculated at final dilutions of 1:100 and 1:1000 onto several cell lines and incubated at different temperatures (see **Table 2** for details). Cultivation was done for seven days, followed by a passage to fresh cells and sub cultivation for another seven days before final observation for cytopathological effects.

Table 2. Cell lines and temperatures used for viral cultivation of organ pools from Siberian sturgeon juveniles.

Cell line	Temperature (°C)
Bluegill fry (BF-2)	15
Common carp brain (CCB)	20
Epithelioma Papulosum Cyprini (EPC)	15
Grunt fin (GF)	20
Snakehead (SSN-1)	20
SSN clone (E11)	20

White sturgeon skin (WSSK)	15 + 20
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RNA was extracted using NucleoSpin® RNA (Macherey-Nagel) according to manufacturer's instruction. RT-qPCR for nervous necrosis virus (NNV) was performed according to the protocol developed by Baud et al. 2015 [11].

DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) according to manufacturer's instruction and qPCR for AcIV-E was performed as previously described [3]. Briefly, targeting the AcIV-E major capsid protein (MCP) gene, the qPCR reaction was performed in 25 µL with 400 nM of each primer (oPVP346 and oPVP347) and 150 nM probe tqPVP20 (Multiplex PCR kit; Qiagen). Reactions were performed on a CFX 96 apparatus (Bio-Rad). The following protocol was adopted: initial denaturation for 15 min at 95°C, 40 cycles of denaturation for 15 s at 94°C and annealing for 60 s at 60°C. A positive control (plasmid kindly provided by dr Laurent Bigarré) as well as negative controls were used in each run.

Confirmatory conventional PCR was performed on samples positive in qPCR. Primers oPVP339—oPVP344 targeting a large segment of the MCP gene was used. Briefly, PCR was performed in 25 µL with 800 nM of each primer, 1.5 mM MgCl₂, 200 IM dNTP and 1,25 U Platinum Taq (Platinum Taq DNA polymerase kit; Invitrogen). The following cycles were applied: 1 step of 5 min at 95°C for Taq polymerase activation followed by 40 cycles at 94°C for 30 sec, 53°C for 30 sec, 72°C for 60 s and a final extension at 72°C for 10 min. A volume of 7 µL end product was run on 7% acrylamide gel at 200V, 400mA, 100 W for 40 minutes.

Sanger sequencing was done according to standard procedures [12]. Sequencing data were assembled using SeqScape v2.5 (Applied Biosystems).

Sampling for bacteriology was done from kidney and ventricle in two fish from the first batch. Samples were inoculated on 5% horse blood agar and Tryptone Yeast Extract Salts (TYES) agar and incubated at 20°C for 5 days.

5. Conclusions

We describe here a severe disease outbreak associated with AcIV-E detection. Stressful transport conditions probably played an important role in exacerbating the mortality. This is another case confirming that iridovirus infections can be associated with severe losses in sturgeon farms. Sturgeon specific viruses are not controlled by legislation, facilitating spread of disease with sub clinically infected individuals and contributing to the already problematic issue of working with sturgeon health.

Supplementary Materials: Video S1: Three live Siberian sturgeon fingerlings showing different signs of viral infection. Lethargy, loss of equilibrium, erratic swimming, buoyancy problems, hyperventilation and emaciation can be seen.

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Conflicts of Interest: The authors declare no conflict of interest

References

1. Ciulli, S.; Volpe, E.; Sirri, R.; Passalacqua, P.L.; Cesa Bianchi, F.; Serratore, P.; Mandrioli, L. Outbreak of mortality in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons associated with sturgeon nucleo-cytoplasmic large DNA virus. *Vet Microbiol.* **2016**, *191*, pp 27–34, DOI: 10.1016/j.vetmic.2016.05.012.

2. Prearo, M.; Pedron, C.; Caruso, C.; Pastorino, P.; Masoero, L.; Burioli, E.; Bigarré, L.; Toffan, A. Acipenser Iridovirus European (AcIV-E) in Russian Sturgeon farmed in Italy: cases description. In: Proceedings of the 18th international conference for the European Association of Fish Pathologists, Belfast, Northern Ireland, 4-7 September 2017. Abstract 068-P, pp 286.
3. Bigarré, L.; Lesne, M.; Lautraite, A.; Chesneau, V.; Leroux, A.; Jamin, M.; Boitard, P.M.; Toffan, A.; Prearo, M.; Labrut, S.; Daniel, P. Molecular identification of iridoviruses infecting various sturgeon species in Europe. *J Fish Dis* **2017**, *40*(1), pp 105-118. DOI: 10.1111/jfd.12498.
4. Clouthier S.; Breyta R.; Kurath G.; Anderson E. Sturgeon nucleo-cytoplasmic large DNA virus phylogeny. Proceeding of the 10th International symposium on viruses of lower vertebrates. Budapest 4-7 June, 2017, pp 58
5. Waltzek T. B. Overview of the nucleo-cytoplasmic large DNA viruses (NCLDV). Proceeding of the 10th International symposium on viruses of lower vertebrates. Budapest 4-7 June, 2017, pp 57
6. Whittington, R.J.; Becker, J.A.; Dennis, M.M. Iridovirus infections in finfish - critical review with emphasis on ranaviruses. *J Fish Dis* **2010**, *33*(2),95-122, DOI: 10.1111/j.1365-2761.2009.01110.x.
7. Watson, L.R.; Groff, J.M.; Hedrick, R.P. Replication and pathogenesis of white sturgeon iridovirus (WSIV) in experimentally infected white sturgeon *Acipenser transmontanus* juveniles and sturgeon cell lines. *Dis Aquat Organ* **1998**, *32*, pp 173-84, DOI: 10.3354/dao032173.
8. Kurita, J.; Nakajima, K. Megalocytiviruses. *Viruses* **2012**, *4*, pp 521-38. DOI: 10.3390/v4040521.
9. Branson, E. Environmental aspects of aquaculture. In *Aquaculture for veterinarians*, 1st ed; Brown, L., Ed.; Pergamon Press Ltd, Oxford, UK, 1993; Chapter 4, pp 57-68, ISBN 0 08 040835 4
10. Pappenheim, A. Die kombinierte May-Grünwald-Giemsa – Färbungsmethode als histologische Universalübersichtsfärbung. *An An* **1912**, *42*, pp 525-527.
11. Baud, M.; Cabon, J.; Salomoni, A.; Toffan, A.; Panzarin, V.; Bigarré, L. First generic one step real-time Taqman RT-PCR targeting the RNA1 of betanodaviruses. *J virol methods* **2015**, *211*, pp 1-7.
12. Abbadi, M.; Zamperin, G.; Gastaldelli, M.; Pascoli, F.; Rosani, U.; Milani, A.; Schivo, A.; Rossetti, E.; Turolla, E.; Gennari, L.; Toffan, A.; Arcangeli, G.; Venier, P. *Gen Virol* **2018**, *99*, pp 693-703, DOI: 10.1099/jgv.0.001042.