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Hematology patterns of migrating European eels and the role of EVEX virus

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Abstract

We show that European eels infected with the rhabdovirus EVEX (Eel Virus European X) virus, developed hemorrhage and anemia during simulated migration in large swim tunnels, and died after 1000–1500 km. In contrast, virus-negative animals swam 5500 km, the estimated distance to the spawning ground of the European eel in the Sargasso Sea. Virus-positive eels showed a decline in hematocrit, which was related to the swim distance. Virus-negative eels showed a slightly increased hematocrit. Observed changes in plasma lactate dehydrogenase (LDH), total protein and aspartate aminotransferase (AAT) are indicative of a serious viral infection. Based on these observations, we conclude that eel virus infections may adversely affect the spawning migration of eels, and could be a contributing factor to the worldwide decline of eel.

Keywords: European eel; *Anguilla anguilla*; Virus; Simulated migration; EVEX; Blood chemistry; Lactate dehydrogenase; Total protein; Aspartate aminotransferase

1. Introduction

Worldwide, eel populations have been dwindling over the last decade. Steep declines of 90–99% have been reported for European eel (*Anguilla anguilla*), Japanese eel (*Anguilla japonica*), and American eel (*A. rostrata*) (Stone, 2003). Eels are very vulnerable to environmental factors because of their complex life cycle. As a catadromic fish species, they migrate several thousand kilometers to their spawning areas. Possible adverse effects on the adults include contamination with PCBs – which are released from fat stores during their long-distance migration (Castonguay et al., 1994) – and infection with the parasitic swim bladder nematode *Anguillicola crassus* (Haenen et al., 1994). Furthermore, diminished fat stores due to insufficient food supplies in the inland waters (Svedäng and Wickström, 1997), blockage of migration routes by power stations and power plants, and over-fishing, are all possible causes (Castonguay et al., 1994). Changes in oceanographic currents may interfere with transport of eel larvae to the European coast, and this too may contribute to the decline in eel populations (Knights, 2003). However, no conclusive evidence on any of these causes has been presented yet (Dekker, 2004).

A factor, that has not received much attention to date, is the worldwide occurrence of eel viruses (van Ginneken et al., 2004). Viruses are known to affect blood-forming tissues in fish, and typically become virulent during stress (Wolf, 1988). In salmon for example, Infectious Haematopoietic Necrosis Virus (IHNV) and Viral Haemorrhagical Septicemia Virus (VHSV), both rhabdoviruses, can affect hematopoietic tissues, leading to severe anemia (Wolf,

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1988). The most prominent cases of rhabdovirus infections in eel populations, described in literature, are infections with EVA (Eel-Virus-America) and EVEX (Eel-Virus-European-X). Both viruses are serologically related (Kobayashi and Miyazaki, 1996). EVA was first discovered in Japan in 1974, in a shipment of American elvers, which had been stocked in Cuba (Wolf, 1988). Another virus, which was isolated in a shipment from France to Tokyo, was named EVEX because of its European origin (Sano et al., 1977). So EVEX was described for the first time in 1977, in the period when the European eel populations started to decline. At this moment it is not known if EVEX is a virus endemic to the European eel population or that it substantially spread over the past 50 years due to aquaculture practices. EVEX virus has recently been observed in several countries worldwide (van Ginneken et al., 2004) in European eel (*A. anguilla*) in the Netherlands, Italy and Morocco, but also in New Zealand longfin eel (*Anguilla dieffenbachi*). In this respect it is worrying that also *Herpesvirus anguillae* is isolated and identified in eel populations all over the world. In cultured eel in Taiwan (Ueno et al., 1992; Chang et al., 2002), in cultured eels in the Netherlands (van Nieuwstadt et al., 2001; Davidse et al., 1999; van Ginneken et al., 2004) but also (this study) in adult European eels from Lake Grevelingen. In the comprehensive study of Jørgensen et al. (1994) elvers and eel of *A. anguilla* were sampled on 306 occasions in Denmark, United Kingdom, France and Sweden several eel viruses were isolated like EVEX, EVA, IPN, and herpes like viruses. This study also supports our view that viruses are widespread in the eel population.

For eels, long-term migration can certainly be considered a major stressful event. Therefore, one may assume that an outbreak of a virus infection in infected individuals can take place during this journey. Based on the work of Schmidt, who caught leptocephali (the larvae of the eel) in the ocean, it is assumed that the spawning grounds of the European eel are 6000 km removed from the European continent in the Sargasso Sea (Schmidt, 1923; Miller and McCleave, 1994). It is generally assumed that the silver eel does not feed during its journey to the spawning grounds, which it reaches 4 to 6 months later (Tesch, 1977; Fricke and Kaese, 1995).

In order to test this hypothesis we simulated the 5500-km journey to the Sargasso Sea in large Blazka swim tunnels of 127 l, comparing virus-positive and -negative European eels.

2. Material and methods

2.1. Rationale of the experiment, selection of the animals

It was initially our intention to simulate the 5500-km migration of European eel (*A. anguilla* L., Anguillidae, Teleostei) to the Sargasso Sea in 22 Blazka swim tunnels. We used silver eel (1500 g; ±85 cm), caught in the Grevelingen (Netherlands) during their seaward migration in September 2000. Fish were kept in seawater (33 ppt) for 1 month before use in the experiment. The recirculation system and swim tunnels were placed in a climatized room with a constant temperature of 15 °C. The water temperature was kept at 14 °C. Animals were kept under constant dark conditions. Of these animals, four, five and four animals stopped with swimming after approximately 500, 1000 and 1500 km, respectively (Fig. 1).

The animals were sampled live, and blood was collected while organs were investigated for virus infections. All animals were infected with the EVEX virus (Eel-Virus-European-X (unknown). This group was called the *Virus-positive group*. Of the swimgroup only the 1000 (N=5) and 1500 km sample (N=4) were used for blood analysis (see Tables 1 and 2).

In a second trial, to simulate the 5500-km migration of European eel to the Sargasso Sea, we used hatchery animals (700–900 g, ±75 cm). The experiments with virus-negative eels from a hatchery were performed in freshwater at a temperature of 19 °C. The animals were sampled alive after 6 months and blood was collected while organs were investigated for virus infections. All animals were virus free. This group was called the *Virus-negative group*. Blood plasma of all animals was investigated afterwards for blood chemistry at the CKCL-laboratory of the Academic Hospital, Leiden University, The Netherlands.

2.2. Blazka swim tunnel

The Blazka swim tunnel has a length of 200 cm, with a diameter of the outer swim tunnel tube of 28.8 cm and a diameter of the inner swim tunnel tube of 19.0 cm. The volume was 127.14±0.90 L (n=5). It was calibrated with a Laser Doppler technique at the Delft Hydraulics Laboratory, Technical University Delft. The experimental set-up is described elsewhere (van den Thillart et al., 2004). The swimming speed of the water in the swim tunnels was set at 0.5 body lengths per second.

2.3. Experimental protocol

The Virus-positive group consisted of one Swim group (N=13), one Rest group (N=13), and one Initial group (N=10). The Swim group was put in Blazka swim tunnels. The Initial and Rest group were kept in flow boxes (Overtoom BV.) of 40 l connected to the same water recirculation system. The Initial group was sampled at the start of the experiment as a zero sample. Both Swim and Rest groups were kept at the same water quality conditions in the tunnels and flow boxes during the experiment. The *Virus-negative group* consisted of one Swim group (N=9), one Rest group (N=12), and one Initial group (N=9). All animals from virus-positive and virus-negative groups were healthy at the start of the experiment. The presence or absence of viruses in all animals was determined after

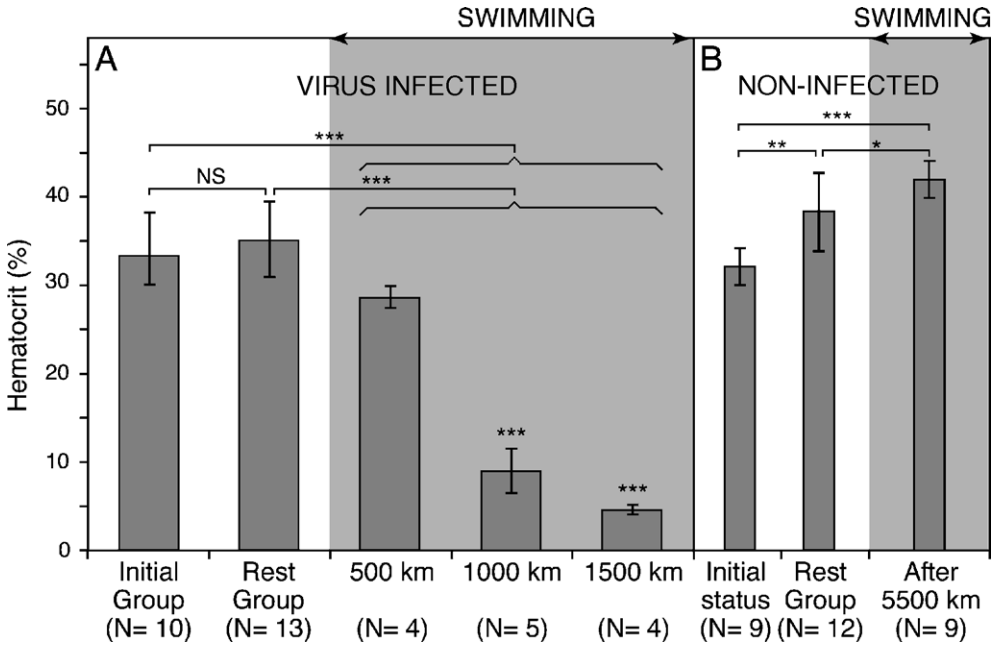

Fig. 1. (A) Hematocrit of European eels, which were found to be infected with EVEX virus. Eels swam for up to 1 month, September 2000, in large 127-l Blazka swim tunnels. The decrease of red blood cells was negatively correlated with the covered distance. (***)—Denotes significantly different $P \leq 0.001$. Control—initially sampled; Rest—1 month rest; (swimming—shaded area): 1 month swimming. (B) Hematocrit of virus-negative eels which swam 5500 km during the period March–September at a continuously speed of 0.5 body length per second in large 127-l Blazka swim tunnels, (swimming—shaded area, 6 months swimming). The hematocrit increased during the 6-month swim period with 11.3 %±4.68 (N=9). Start—initial sample in March 2001. After 5500 km—end sample in September 2001 taken from the same animals.

sampling and dissection of the organs. The animals were not fed during the entire experimental period.

2.4. Sampling and virus isolation

As soon as the animals stopped swimming, they were quickly anaesthetized with 300 ppm MS222 (3-amino-benzoic-acid-ethyl-ester methanesulfonate salt; Sigma, St. Louis, USA). After 3 min, the anaesthetized fish were taken out of the swim tunnel and blood was collected with a heparinized syringe (flushed with 3000 units heparin per milliliter of blood). The spleen, gills, kidney and liver were removed for virus isolation by the Fish Diseases Laboratory (CIDC—Lelystad), and stored on dry ice. Samples of organs were homogenized with sterile sand in sterile medium, and tested on three different cell lines: RTG-2—rainbow trout gonad cells; FHM—fat head minnow cells; and EK-1—eel kidney cells, at 15 °C, 20 °C, and 26 °C respectively. In the case of virus infections, the infected cell line was inspected by electron microscopy followed by either immunofluorescence or immunoperoxidase methods in order to identify the virus type (Wolf, 1988).

2.5. Analytical methods for blood samples

Blood was centrifuged at 8000×g for 5 min. The plasma was aliquoted and stored at −80 °C pending analysis at the CKCL-laboratory of the Academic Hospital, Leiden University, The Netherlands. Lactate dehydrogenase (LDH; EC 1.1.1.27), total protein and aspartate aminotransferase (AAT; EC 2.6.1.1) were measured using a Hitachi 747 analyzer (Roche, Almere). LDH was measured according to the

Table 1
Test results (mean±SD) among healthy (virus-negative) animals for the parameters body weight, length, hematocrit, aspartate aminotransferase (AAT), lactate dehydrogenase (LDH) and total protein

Parameter	Swim group (N=9)	Initial group (N=9)	Rest group (N=12)	P-value Kruskal–Wallis	P-value Sw/In	P-value Sw/Re	P-value In/Re
Body mass (g)	914.7±58.37	688.8±113.4	676.4±70.94	0.0001	0.001	0.001	NS
Length (cm)	74.9±3.17	67.2±5.34	70.88±3.76	0.009	0.002	0.008	NS
Hematocrit (%)	42.3±2.1	32.1±2.0	38.64±4.75	0.0095	0.002	0.040	0.014
AAT (units/ml)	94.7±37.59	106.1±37.56	64.42±34.67	0.001	NS	0.070	0.009
LDH (units/ml)	1300.1±818.3	507.4±264.8	1381.2±1073	0.01	0.014	NS	0.032
Total protein (g/l)	42.0±12.95	38.90±6.80	43.83±3.21	0.218	NS	NS	NS

Comparison between groups: Swim–Initial (Sw/In), Swim–Rest (Sw/Re) and Initial–Rest (In/Re). NS indicates P-value ≥0.1.

Table 2
Test results (mean±SD) of infected (virus-positive) animals for the parameters body mass, length, hematocrit, aspartate aminotransferase (AAT), lactate dehydrogenase (LDH) and total protein

Parameter	Swim group (N=14)	Initial group (N=10)	Rest group (N=13)	P-value Kruskal–Wallis	P-value Sw/In	P-value Sw/Re	P-value In/Re
Body mass (g)	1458.5±175.5	1433.8±233.1	1652.3±354.7	0.564	NS	NS	NS
Length (cm)	85.4±8.7	87.85±3.94	89.44±7.34	0.579	NS	NS	NS
Hematocrit (%)	7.03±4.3	32.32±7.34	34.01±7.99	0.001	0.002	0.002	NS
AAT (units/ml)	2175±2510	98.83±91.84	156.9±126.9	0.0001	0.001	0.001	NS
LDH (units/ml)	7290.9±5447.9	1771±921.3	625.9±775.0	0.0001	0.006	0.001	0.005
Total protein (g/l)	24.19±12.42	44.70±5.01	41.3±4.47	0.0001	0.001	0.002	NS

The units/ml for LDH and AAT correspond to μmol NADH/ml/min oxidized to NAD+ at 37 °C. Comparison between groups: Swim–Initial (Sw/In), Swim–Rest (Sw/Re) and Initial–Rest (In/Re). NS indicates P-value ≥0.1.

method of the German Society for Clinical Chemistry (1972); aspartate aminotransferase according to IFCC method without pyridoxal phosphate (Klauke et al., 1993); total protein was based on the Biuret method (Camara et al., 1991). Hematocrit values were measured in 9-μl whole blood samples using an hematocrit micro-centrifuge (Bayer, Germany).

2.6. Clinical significance of blood chemistry parameters

We selected a set of parameters, which are used in human pathology as a diagnostic tool for virus infection (Burtis et al., 1996). The AAT activity was determined to estimate the liver function. Furthermore, we used two parameters as indicators for liver disorder and/or general catabolism: (a) total plasma protein and (b) lactate dehydrogenase (LDH).

2.7. Statistics and calculations

Mean±standard deviations are given in the Tables 1 and 2. For all three groups Initial, Swim and Rest, the mean value of every measured parameter was compared pairwise. A Kruskal–Wallis test was performed on the data to check for significant differences between the three groups. Further statistics were performed using a one-way ANOVA. Comparisons of mean squares of the ANOVA were tested using F-tests. $P \leq 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov and F_{max} tests, respectively.

3. Results

In this study, EVEX virus, a rhabdovirus, was detected in Grevelingen animals which swam for a mean time period of 28.6±7.7 days (range 14.0–40.0 days) and had covered a mean distance of 1048±278 km (range 526–1521 km). The mean distance covered was 36.9±3.6 km/day (range 26.4–40.2 km). EVEX-infected eel may show anemia, with blood in the abdominal fluid and hemorrhage all over the body. While the resting Initial group showed no signs of infection, the Swim group developed severe hemorrhage and anemia at various time points, which apparently forced them to stop swimming (Fig. 1A).

All animals in Initial, Swim and Rest groups of the Grevelingen animals were infected with the EVEX virus although the drop in hematocrit was only noticeable in the Swim group. The animals in the virus-negative Swim group swam for a period of 173 days and covered a mean distance of 5533±342 km (range 4900–5949 km). The mean distance covered was 31.98±2.05 km/day (range 28.3–34.4 km). In the group of non-infected hatchery animals the opposite effect was observed. The initial hematocrit value of 32.1±2.0% increased after 5500 km to 42.3±2.1% (Fig. 1B). To examine our hypothesis that a virus infection is the reason for the collapse, we tested plasma samples for LDH, total protein, and AAT (Tables 1 and 2).

In Table 1 (healthy, virus-negative) and Table 2 (infected, virus-positive) the effect of swimming, the time aspect (endurance of the experiment with starving animals) and the influence of the virus was assessed.

In the healthy group, swimming results had no effect on any of the selected parameters (Table 1). In the healthy group, a time effect results in a significant difference for AAT and LDH between Initial and Rest group. In the virus-infected group this is only the case for LDH between Initial and Rest group. A clear virus effect can be observed in the infected Swim groups resulting in strongly significant different values of AAT, LDH and total protein in comparison with Initial and Rest group.

4. Discussion

In this study EVEX virus was detected in a group of European eels used for long-term swimming experiments. While the Rest group showed no signs of illness and looked healthy, the Swim group showed signs of hemorrhage and severe anemia at different time points, which apparently forced them to stop swimming (Fig. 1A). The hemorrhage took the form of petechial hemorrhages all over the body, and bloody abdominal fluid. Wolf (1988) described these defects as obvious signs of a severe viral infection.

EVEX is the most prominent rhabdovirus, infecting eel populations (Jørgensen et al., 1994, van Ginneken et al., 2004). Recently we found EVEX in European eel collected across its natural range: The Netherlands, Italy and Morocco. The virus was also observed in New Zealand longfin eel (*A. dieffenbachi*). In contrast, glass eel collected from eel farms in The Netherlands were mainly infected with HVA (*H. anguillae*).

Fish production via aquaculture has more than doubled in weight and value between 1986 and 1996 and over one quarter of human fish consumption is produced in aquaculture (Naylor et al., 2000). With the growing amount of in aquaculture produced products, transfer of diseases by transport of stock and food supplies has increased. Blanc (1997) points out that nearly one hundred pathogens have been introduced in European hydrosystems since the introduction of aquaculture. Widespread infection of the eel population with for instance EVEX virus may result from unlimited intercontinental transport. In a recent survey we found many viruses in eel populations in The Netherlands (van Ginneken et al., 2004), which threatens for the whole eel population as The Netherlands is one of the leading eel-trading countries (Heinsbroek and Kamstra, 1995).

We therefore hypothesize that virus infections, probably easily spread due to increasing proximity with humans, may be a contributing factor to the worldwide decline of eel populations.

We compared infected and non-infected animals for their spawning migration in large swim tunnels in the laboratory. Also we measured blood biochemistry data like hematocrit, total protein, AAT and LDH. We have to acknowledge that we used in this study eels of different life history stages. The infected eels were silver (migratory stage), from a wild brackish water population, and with a size of around 1500 g. The non-infected eels were yellow (sedentary stage) originating from a hatchery run on freshwater and with a size of around 700–900 g. The rationale behind this possible flaw in the experimental set-up was that the wild eel population seemed to be infected with the EVEX virus (van Ginneken et al., 2004). We were afraid to loose the eels in an early stage due to the EVEX virus, as for three consecutive years we tried to have a 5500-km simulated migration run with Grevelingen eels in the swim tunnels. Every time the animals collapsed after 500–1500 km. Eel farmers immunize their eels against eel viruses by means of bath exposure at the glass eel (elver) stage to adult, virus positive eels (personal communication with Ir.J.van Rijsingen, Royaal BV., Helmond, The Netherlands).

We choose virus-resistant hatchery eels on freshwater for the 5500-km run. To our opinion this difference between the two eel groups had no major impact on blood chemistry parameters. In the Rest group there was (except for AAT) no significant difference between Grevelingen (infected) and hatchery (non-infected) animals giving *P*-values for total protein, AAT and LDH of respectively, $P \leq 0.291$, $P \leq 0.019$*, $P \leq 0.052$. In contrast in the Swim group the *P*-values between Grevelingen (infected) and hatchery (non-infected) animals for total protein, AAT and LDH were highly significant, respectively, $P \leq 0.009$**, $P \leq 0.001$**, $P \leq 0.002$**. This indicates that the initial status of the animals remained the same but that due to swimming exercise in combination with a virus infection there was a shift in blood chemistry parameters.

Here, we have shown that LDH levels increased in the infected Swim group. LDH is an abundant enzyme present in all tissues and is released upon tissue damage. Increased levels of LDH are associated with hemolysis, liver disease or hypoxemia due to a severe shock or anoxia (Burtis et al., 1996). Reduced total protein levels in the infected Swim group suggest bleeding and/or liver failure. In principle, total plasma protein can be used as an indicator for disease for two reasons. First, most plasma proteins (with the exception of the immunoglobulins and protein hormones) are synthesized in the liver (Burtis et al., 1996). Therefore changed protein plasma levels may point to liver failure. Secondly, hemodilution can cause decrease of proteins. AAT is significantly higher in the infected Swim group. A small increase is normal after muscle exercise, but in this case the significantly increased level of AAT may suggest liver damage, hemolysis or general tissue damage. All three parameters, increased LDH, decreased total protein and increased AAT, are consistent with the hemorrhage seen in the animals. Possibly, the anemia is the result of internal blood loss, since the symptoms include blood in the abdominal fluid and hemorrhage all over the body.

Acute human hepatocellular injury, whether due to viral hepatitis, hepatic ischemia or drug hepatotoxicity also resulted in elevated levels of serum AAT, while LDH was increased in case of ischemic hepatitis. It was concluded that these parameters may be helpful in the differential diagnosis of acute liver injury (Cassidy and Reynolds, 1994).

The second long-term swim trial of 5500 km with the healthy virus-negative group showed the opposite effect: the initial hematocrit value of 32.1% (±2.0) increased after 5500 km to 42.3% (±2.1) (Fig. 1B). Normal hematocrit values for healthy yellow vs. silver eel are 26.5±1.0% and 36.4±1.2%, respectively (Johansson et al., 1974).

For humans, long endurance exercise, resulting in a training effect with increased hematopoiesis, can probably be associated with the function of the hormone erythropoietin (Leigh-Smith, 2004). The expression of an erythropoietin-like gene has been described in fish (Shiels and Wickramasinghe, 1995). So, it is likely that, the increased hematocrit in the group that swam 5500 km could be mediated by erythropoietin.

Infection with EVEX, and anemia in healthy-looking adults, may have serious consequences for the population. As a catadromic fish species, eels have a very complex life cycle. The return journey of the adults of 5500 km to their spawning grounds may be considered a stressful situation. If the changes observed in blood profile for European eel

during a migration of 5500 km in large swim tunnels in the laboratory also happen in EVEX-infected eels in the ocean, this may have serious consequences for the adult silver eel during their natural migration.

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