



Experimental models of ecological niches for African swine fever virus



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ABSTRACT

In this study, we investigated the possible biological factors affecting the survival of the African swine fever virus (ASFV) in the environment and their potential to influence the ecology of the ASFV. Specifically, we tested the survival and replication of ASFV in four phylogenetically distinct organisms: *Paramecium caudatum*, *Dendrobaena alpine*, *Aedes aegypti* and *Xeropicta derbentina* using qReal-Time PCR and hemadsorption analysis. Levels of ASFV in earthworms (*Dendrobaena alpina*) and soil declined at similar rates, suggesting that earthworms likely have no influence on the ecology of the ASFV. Ciliates (*Paramecium caudatum*) significantly increase the rate of ASFV disappearance from the aquatic environment, probably using the virus as a food source. Mosquitoes (*Aedes aegypti*) do not provide significant support for the persistence of ASF virus in the environment, with no evidence for transmission to their offspring or pigs that ingested mosquitoes. ASFV persisted for much longer in air-breathing land snails (*Xeropicta derbentina*) than in the soil. Moreover, transcription of viral genes was maintained within the snail, although the question of full-fledged viral replication is still open. In addition, the active movements of snails suggests that they could play a role in the spread of the virus. The virus is likely to be localized in the intestines of snails as it is regularly excreted from their feces. These results highlight the importance of investigating invertebrates for understanding ASFV surviving, spreading and transmission in natural populations with zoonotic transmission potential.

1. Introduction

African swine fever virus (ASFV) is a large enveloped double-stranded DNA virus with several unique characteristics and differs from other viruses. ASFV is the sole member of the genus *Asfivirus* within the family *Asfarviridae* and is the only known DNA arbovirus that causes diseases ranging from acute and fatal to chronic or even asymptomatic infections. Due to the lack of a vaccine or effective antiviral treatment, it is important to comprehend the transmission and maintenance pathways to prioritize future ASFV research on vector ecology and aid with socio-economic stability, proactive prevention, detection, and response tactics.

Outside Africa, the routes of transmission of African swine fever (ASF) virus are mainly through direct contact between infected and susceptible animals and/or through contaminated animal feed. It is well known that pathways of ASFV transmission include pig to pig contact, contaminated secretions (blood, feces, urine, mucus), fomites (vehicles,

equipment), aerosols and through the bite of Ornithodoros soft ticks, which are competent vectors (O'Neill et al., 2020; Pereira de Oliveira et al., 2019; Golnar et al., 2019). Furthermore, there is evidence of seasonality in outbreaks in some pig farms in Europe and Asia (Liu et al., 2020; O'Hara et al., 2020; Pautienius et al., 2020). ASFV also occurs in wild boar and it is important to understand the role of climate, land cover, habitat quality and human activity (Guberti et al., 2019). This persistence of ASFV in these environments suggests the possible participation of new vectors in viral transmission.

The nature of the relationship of arboviruses with vertebrates and its effect on the formation of the circulation cycles of these viruses first was studied using models of bunyaviruses (Jonkers et al., 1968), alphavirus (de Moor and Steffens, 1970), and flaviviruses (Boshell et al., 1968). In all cases, it was shown that only those vertebrates that develop viremia exceeding the threshold level of viral infectivity for specific vectors are involved in maintaining the natural circulation cycles of these viruses. Even though the ASF virus continues to circulate in Eurasia, to date, no

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such systems have been identified that contribute to the preservation of the virus in natural conditions. Therefore, it can be assumed that the circulation of the virus is associated with the possibility of long-term survival in any ecological niche.

Knowledge about possible arthropod vectors of ASFV, except Ornithodoros soft ticks, is limited (Forth et al., 2018). Potential ASFV transmission pathways through arthropods (soft and hard ticks, Culicoides, mosquitoes, biting flies, sand flies, lice, and fleas) have been investigated previously. Basically, the data of various authors indicate the possibility of mechanical transmission of the virus through the bites of blood-sucking insects. Usually, this transmission of the pathogen determines the short survival time of the virus in mechanical vectors (Mellor et al., 1987; Olesen et al., 2018). Some positive detection were obtained from biting flies, which can be involved in the mechanical vector pathway, as well as from hematophagous arthropods, which may effectively contribute to the speed of virus transmission, but it is likely that this role is of lesser importance compared to transmission mechanism between Suidae (Bonnet et al., 2020). Although mosquitoes are quite versatile carriers of many viruses, there is a lack of scientific data on the potential role of mosquitoes as biological or mechanical vectors in ASFV transmission. A few studies, conducted in natural conditions, showed the absence or low levels of virus in mosquitoes (Herm et al., 2020, 2021; Qin et al., 2021; Bonnet et al., 2020). Moreover, in natural conditions, finding the ASFV genome in insects can represent general environmental contamination with the virus (Herm et al., 2020). The data obtained in these studies, therefore, cannot confirm the role of mosquitoes in ASFV transmission.

Despite of the above, special of interest are Protozoa, and in particular ciliates that can play an important role in viral epidemiology and such actions are beginning to be understood. Protozoa may decrease virus levels by grazing virus particles. In the marine environment, some protozoans were found to ingest viruses (Gonzalez and Suttle, 1993). The interaction between ciliates and ASFV are important because they can be found in most freshwater habitats, including engineered ones and could play important role in the ecology of a virus caught in the water.

Within this study, we used four common and phylogenetically distinct organisms: *Paramecium caudatum* (Family *Parameciidae*, Order *Peniculida*), *Dendrobaena alpine* (Order *Opisthopora* Suborder *Lumbricina*), *Aedes aegypti* (Family *Culicidae*, Order *Diptera*), *Xeropicta derbentina* (Family *Hygromiidae* Superfamily *Helicoidea*).

2. Methods

2.1. Virus

We used African swine fever virus (Armenia08 strain) in all experiments. ASFV was obtained from spleen of infected pigs. The virus (Armenia08) was first isolated in 2007 from the spleen of an ASFV infected swine.

Virus titration was performed as described previously and expressed by hemadsorption - as lg₁₀ HAD₅₀/mL for non-adapted cells (Enjuanes et al., 1976). The titer was expressed as hemadsorption units - a HADU50/mL. HADU technique was performed on primary culture of porcine alveolar macrophages (PAMs). For PAM obtaining 3 month aged piglets were euthanized and the lungs removed. Cells obtained during bronchoalveolar lavage (BAL) were resuspended in sterile Hank's balanced salt solution. They were centrifuged at 600 g for 10 min and resuspended in RPMI 1640 with 5% fetal bovine serum (FBS) at an initial cell concentration of 3 × 10⁵ mL. After 3 h at 37 °C in a humidified, CO₂ incubator, the adhered cells (porcine alveolar macrophages- PAMs) were washed three times with RPMI to remove contaminating non-adherent cells and then reincubated in RPMI 1640 with 10 % FBS (Forman et al., 1983).

Inactivation of ASFV was done with incubation in water bath (65 °C at 10 min). After heat inactivation virus tested for infectivity.

2.2. Water

Tap water (10 mL) spiked with 1.0 mL of infectious spleen extracts (6.5 lg₁₀ 50 % hemadsorbing doses (HAD₅₀)/mL of African swine fever virus (ASFV) "Armenia08") was stored in glass containers and examined at 23–27 °C (B). Initial dose of virus was 5.5 HAD₅₀/mL.

2.3. Soil

Yard soil (1000 g) spiked with 100 mL of infectious spleen extracts (6.5 lg₁₀ 50 % HAD₅₀/mL of African swine fever virus (ASFV) "Armenia08") was stored in plastic containers and examined at 23–27 °C (B). Initial dose of virus was 5.5 lg₁₀ HAD₅₀/g. The pH range was 7.9–8.1.

2.4. Colony maintenance of *Paramecium caudatum*

Paramecium caudatum (*P. caudatum* Family *Parameciidae*, Order *Peniculida*) was isolated from local pools and single cell clones were prepared. In our study, we used ciliated protozoa *Paramecium caudatum* isolated from well growing laboratory clones (011R4). Cell number per mL was calculated as described previously (Duncan et al., 2011). Paramecia were cultured in physiological, inorganic Losina-Losinsky salt solution Losina-Losinsky (1931) supplied with *Saccharomyces cerevisiae* and bacterium *Klebsiella pneumoniae* as digestive resource at room temperature 22–24 °C which is near the optimum reported for paramecia Wichterman (1986).

The population of *P. caudatum* was counted daily using a Sedgwick-Rafter cell counting chamber at magnification 8x.

2.5. Cocultivation of African swine fever virus and *Paramecium caudatum*

Water (10 mL) with *Paramecium caudatum* (100 ciliatae per mL) spiked with 0.1 mL of infectious spleen extracts (6.5 lg₁₀ 10 hemadsorbing doses (HAD₅₀)/mL of African swine fever virus (ASFV) "Armenia08") was stored at 22–24 °C.

Inactivation and destruction of *P. caudatum* took place by freezing and thawing the samples three times. The number of destroyed ciliate was counted before freezing and thawing starting from the average number of *P. caudatum* in the control group – 50 cells/mL.

Thermal inactivation of the virus was carried out in similar extracts of the spleen. Coincubation of *P. caudatum* with the inactivated virus was carried out under the same conditions as with the live virus.

2.6. Transmission of African swine fever virus to earthworm *Dendrobaena alpina*

The earthworm *Dendrobaena alpine* (Order *Opisthopora* Suborder *Lumbricina*) (Rosa, 1893), cited from Szederjesi (2017), about 6 months old (body weight 1 g) was obtained from a vermiculture plant and allowed to grow and adapt in soil conditions for up to 80 d before use in experiments. The earthworms were kept alive in moist soil in dark conditions. Environmental conditions were maintained at 22–24 °C and 70–80 % humidity (Das and Osborne, 2017).

The earthworms spiked with infectious spleen extracts (n = 80) by injection in dose 0.1 mL/g. The dose of ASFV was 5.5 lg₁₀ HAD₅₀/g. For injections were used standard insulin syringes with needles 29 gauge in diameter.

2.7. Colony maintenance of *Aedes aegypti*

Mosquitoes are held at 30 ± 0.5 °C and 50–70 % relative humidity, with a 12:12 h (light:dark) photoperiod. For the larval rearing eggs were fed with ~300 mg of fish food and a few grains of active dry yeast to induce hatching. Routine colony maintenance of *Aedes aegypti* was done

according to (Ross et al., 2017).

2.8. Transmission of African swine fever virus to mosquitoes

The feeding process of female mosquitoes with virus free blood was performed on the arm of the researcher (Fig. 1A). Artificial feeding was used to introduce the ASF virus to *A. aegypti* (Family Culicidae, Order Diptera) females (Fig. 1B). Eggs of *A. aegypti* collected 3–7 days after artificial feeding with blood containing ASFV (Fig. 1C) and were used for the detection of ASFV. Mosquito excreta samples were collected 0.5–5 hours after feeding (Fig. 1D) on filter paper and frozen at -20 °C.

ASFV with a titer of 5.5 lg 10 HADU₅₀/mL was used to infect mosquitoes. The virus (obtained from porcine spleen) was mixed with researchers' fresh human blood in the ratio of 1:10 and then fed to mosquitoes with the help of a feeder using a pig's intestine as a membrane. Thus, the dose of the virus in the blood used to feed the mosquitoes was 10^{4.5} HADU₅₀/mL. Viral doses were adjusted to levels of the virus in the blood in the acute form of ASF. Female All female mosquitoes (in group which will be fed later with infected blood; 14 days old, n = 30) were starved for 24 h before feeding with infected blood. All mosquitoes from this group were collected and investigated for virus detection. All analyses were performed at 1 (n = 4), 4 (n = 4), 7 (n = 4), 14 (n = 4), 20 (n = 4), 25 (n = 4) and 30 (n = 4) days post infection (total n = 28) (dpi) for all female mosquitoes at 29–32 °C. Blood-fed mosquitoes from 1 (n = 4), 4 (n = 4), 7 (n = 4), 14 (n = 4), 20 (n = 4), 25 (n = 4) and 30 (n = 4), days of investigation were sampled, and virus titers were evaluated by qRT-PCR and HADU₅₀/mL (all data was rescaled to milliliters and grams). Another group of female rest of the mosquitoes (n = 30) were maintained until oviposition and investigated after it (day 10). Eggs from next generation (random selected n = 20) was investigated to the presence of virus by qRT-PCR and HADU₅₀/mL.

Larvae from next generation (random selected n = 15) also was investigated to the presence of virus by qRT-PCR and HADU₅₀/mL.

For comparison, non-sterile tap water with a virus at a dose of 4.5 lg HADU₅₀/mL was used as a control.

2.9. *Xeropicta derbentina* cultivation and infection

In this study, adult snails of mixed sex (shell length = 1.5 cm, body weight range was 0,474–0,629 g) of *Xeropicta derbentina* (Family Hygromiidae Superfamily Helicoidea) were used. All the snails were obtained from the laboratory reared culture originally from field collections in Yerevan, Kanaker. Identification of snails was done according to their shell morphology (Köhler et al., 2009).

The snails were infected using a virus diluted in water (6.5 lg 10 HADU₅₀/mL). To do this, the snails were placed in a tank with infected water a low water level. The soil samples identical to soil where the snails were habitat also investigated as control (5.5 lg 10 HADU₅₀/mL). Virus levels were studied by destroying two mollusks and collection of feces beginning on the 1 st day post-infection (dpi). The investigations of the virus amount in the soil and in the samples were carried out every 5 days.

2.10. Gene expression analysis by quantitative real-time PCR

qReal-Time PCR analysis was used to quantify the isolated DNA of the virus in mosquitoes after artificial feeding and the presence of infectious particles measured by HADU₅₀/mL.

ASFV expression in PAM cell lines, total viral RNA/DNA was isolated using the HiGene™ Viral RNA/DNA Prep Kit (BIOFACT). RNA/DNA samples were then reverse transcribed with REVERTA-L kit (AmpliSens Biotechnologies). Both methods were done following the manufacturer's instructions. We also included a negative control (mosquitoes fed blood without ASFV). Quantitative real-time PCR was performed using the SYBR green methods as previously described (Yin et al., 2001; Ginzinger, 2002) on an Eco Illumina Real-Time PCR system device (Illumina Inc). Each reaction mixture (20 µL) was composed of 4 µL of 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne), 0.2 µL of each specific primer, 4 µL of template DNA /cDNA, and 11.6 µL of ddH₂O. Reactions were carried out in the following conditions: polymerase activation: 95 °C for 12 min, 40 cycles: 95 °C for 15 s, 52 °C for 30 s, and 72 °C for 30 s. Standard curves were created using serial

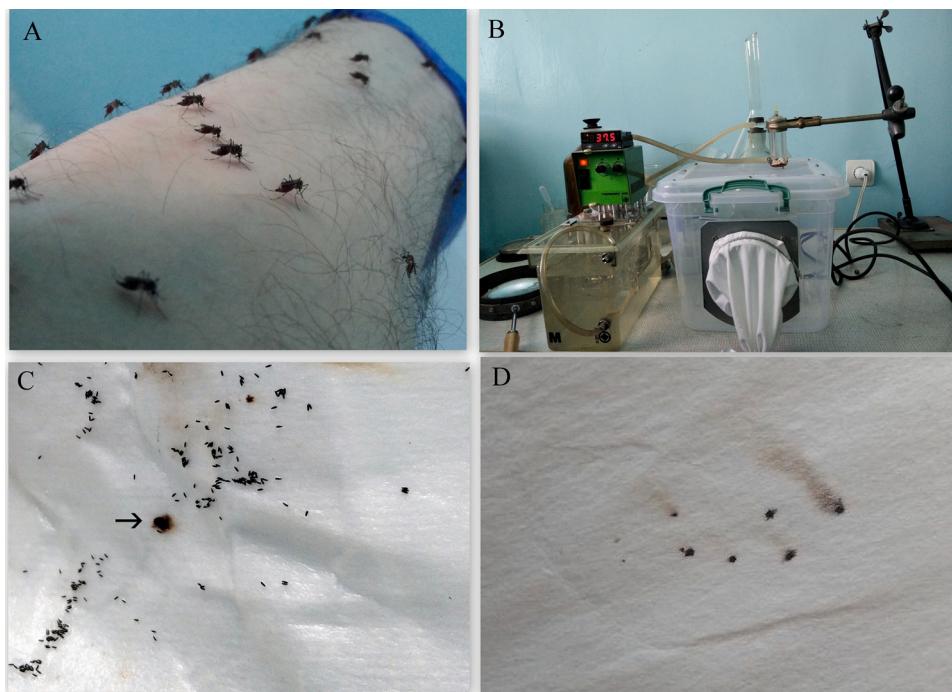


Fig. 1. *Aedes aegypti* feeding and sampling. A. Mosquitoes feed on the arm with ASFV free blood. B. Artificial feeding apparatus using jacketed glass and circulating water bath to maintain the blood infected by ASFV warm (water temperature 37.2-37.5 °C; blood temperature 36.9-37.2) C. Eggs and fluid excretions (arrowed). D. Excreted fluid samples obtained from infected *A. aegypti* females on filter paper used in ASFV detection.

10-fold dilutions of viral DNA. The fluorescence threshold value (C_t) was calculated using the ECO-Illumina system software. Primers used for amplification were designed and ordered from Integrated DNA Technology-IDT (<https://www.idtdna.com/pagesas> follows), K196R (Thymidine kinase gene) F: 5'-GCAGTTGTCGTAGATGAAG-3' and R: 5'-CGAAGGAAGCATTGAGTC3'.

2.11. Transmission of African swine fever virus in the process of natural feeding

Six pigs (Large White breed) were used for investigation of possible transmission of ASFV in the process of natural feeding. Three pigs were fed a diet containing bodies of snails. Three pigs were fed with a diet containing bodies of ASFV infected female mosquitoes.

2.12. Statistical analysis

All virus quantifications were conducted in triplicate. The

significance was evaluated by Mann-Whitney U test for non-parametric values; P values < 0.05 were considered significant. SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

2.13. Ethics approval

All experiments were done according to Institutional Review Board/Independent Ethics Committee of the Institute of Molecular Biology of National Academy of Sciences, Yerevan, Armenia; IRB00004079, 2013

3. Results

3.1. Stability and recovery of ASFV from water with *Paramecium caudatum*

Cultivation of ciliates with the virus led to a slight increase in their size (Fig. 2A;B). However, these differences are not significant (Mann-

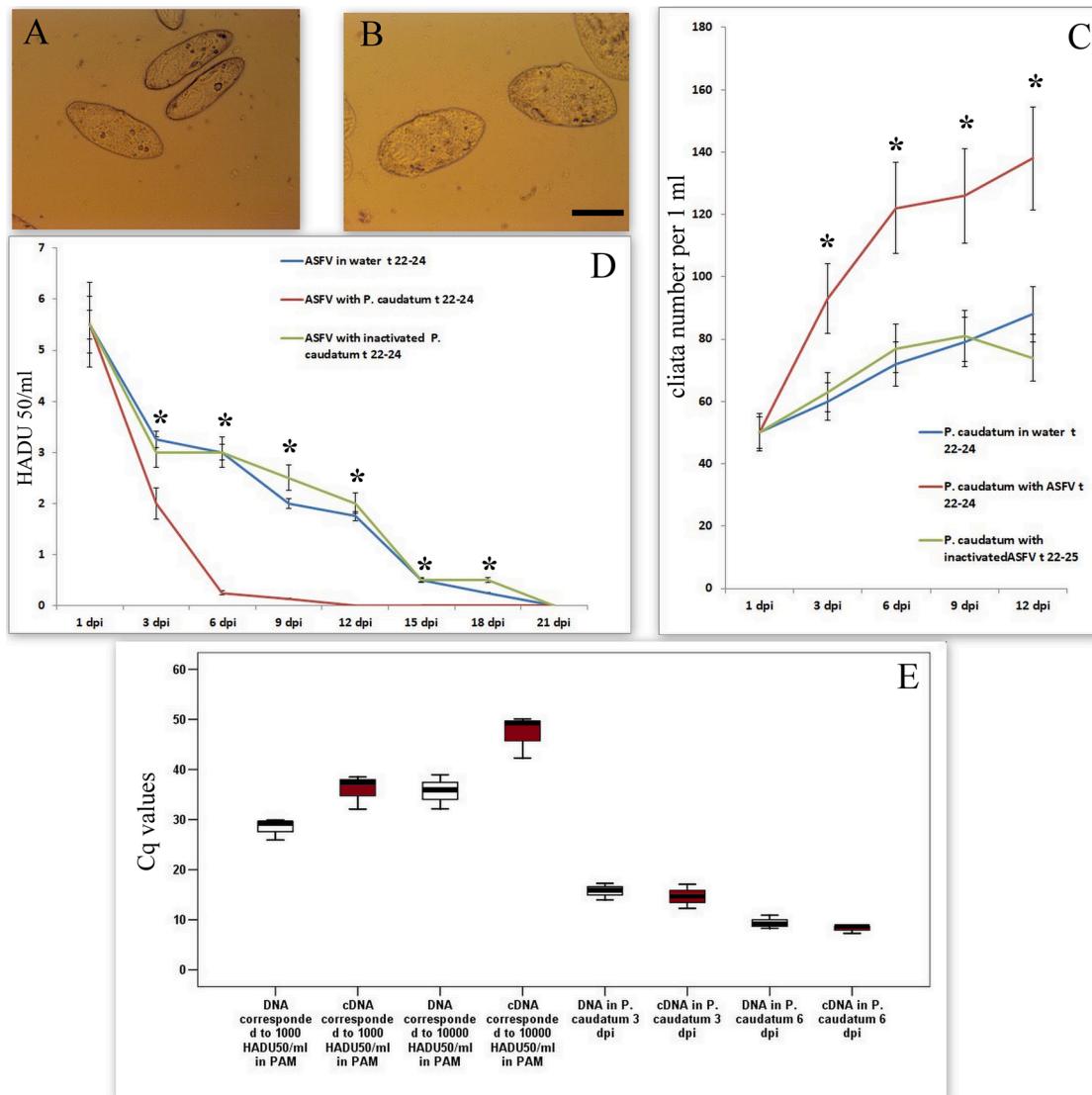


Fig. 2. Cocultivation of ciliate *Paramecium caudatum* and African swine fever virus (Armenia08). A. *Paramecium caudatum* cocultivated with infected spleen extracts with inactivated ASFV (5.5 lg 10 HADU₅₀/mL). *Paramecium caudatum* cocultivated infected spleen extracts with alive ASFV (5.5 lg 10 HADU₅₀/mL). Scale bar 100 μm. C. Decreased levels of ASF virus in water without *Paramecium caudatum*, in environmental water infected by ASFV with *Paramecium caudatum*, and in water with inactivated *Paramecium caudatum* in units of hemadsorption (lg). D. *Paramecium caudatum* amounts in water with alive ASFV (5.5 lg 10 HADU₅₀/mL) and without virus. E. Levels of DNA and transcripts of ASFV K196R gene (expressed in Cq) in water without *Paramecium caudatum* during experiment. *Significant compared to control levels ($p < 0.05$ by Mann-Whitney U test).

Whitney U: $P > 0.05$). Co-cultivation of ciliates with the virus leads to a significant acceleration of the proliferation of *P. caudatum* in comparison with cultivation with normal feeding (Fig. 2C). Co-incubation of *P. caudatum* with inactivated virus shows insignificant difference of the proliferation of *P. caudatum* compared with infection virus (Fig. 2C). Virus titers in water spiked with infectious spleen extracts and stored at 22–24 °C decreased within the first 3 dpi (Fig. 2D) but then leveled out, with the difference between 3 and 6 dpi being non-significant. A constant decrease in the titers of the virus in water continued until complete disappearance at 2–3 weeks.

When the virus is co-cultivated with *P. caudatum*, the decrease in viral titers occurs at a faster rate. The difference in the titers of the virus becomes significant already by 3 dpi, and by 6–9 days with co-cultivation with *P. caudatum* part of the samples was found to be free of the virus. By 12 dpi, no virus was detected in any sample.

Co-incubation of ASFV with destroyed (by freeze/defreeze process)

P. caudatum shows insignificant difference between ASFV titers in control and destroyed cells groups (Fig. 2D).

qRealTime-PCR data revealed similar findings (Fig. 2E). In several parts of the samples, the virus was not detected starting from 6 dpi. Transcriptional activity (increase in cDNA level) was not recorded in any sample, regardless of the cultivation period.

3.2. Stability and recovery of ASFV from earthworms and transmission across generations

In this experiment, the soil with earthworms which were spiked with spleen extracts from ASFV-infected pigs and stored for up to four weeks at 22–24 °C (Fig. 3A;B). A control soil only with ASFV containing spleen extracts were included under the same conditions.

Fig. 3C shows that the number of hemadsorbing units of the virus in the earthworms after injection is the same as the amount of the virus in

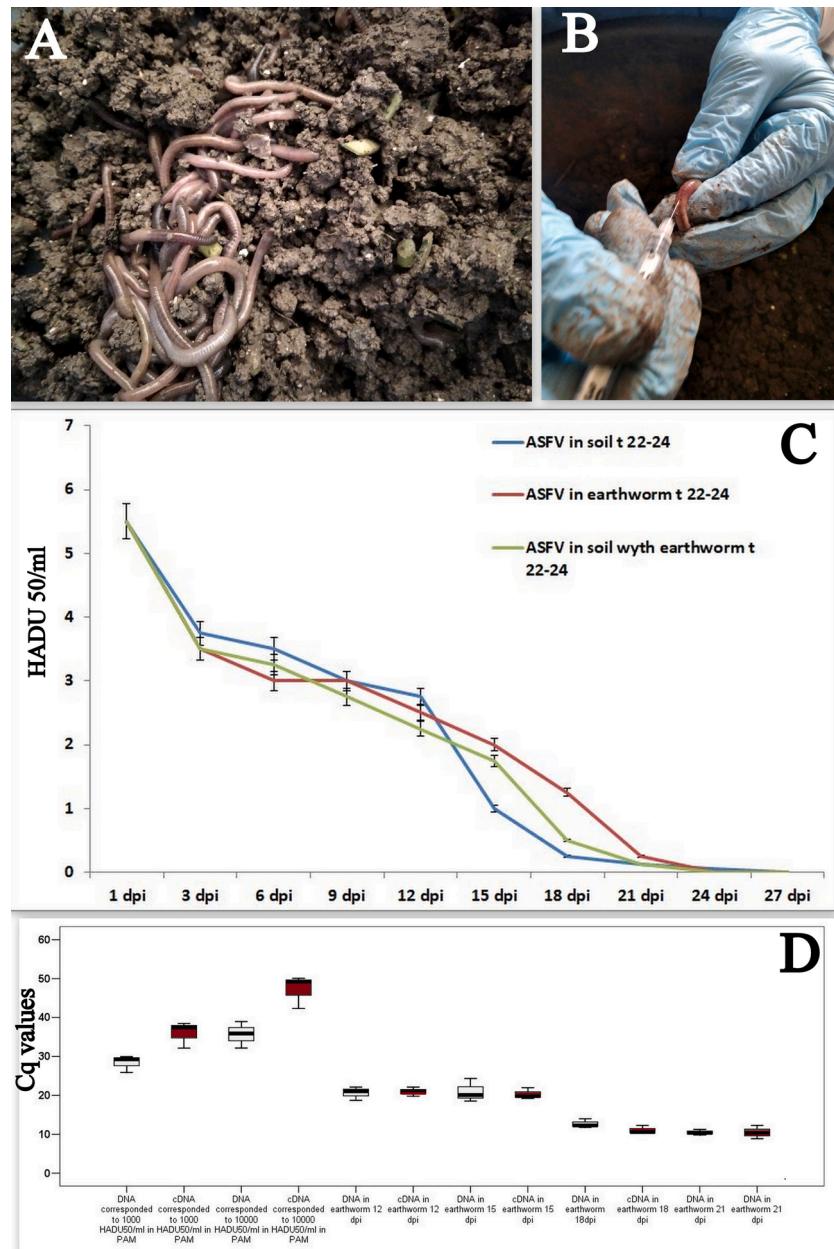


Fig. 3. Cocultivation of earthworm *Dendrobaena alpine* and African swine fever virus (Armenia08). A. Earthworm *Dendrobaena alpine* in experimental incubation in container. B. Introduction of ASFV to earthworm *Dendrobaena alpine* via injection. C. Levels of ASF virus in earthworm's bodies, in soil infected by ASFV without *Dendrobaena alpine* (lg). D. Levels of DNA an transcripts of ASFV K196R gene (expressed in Cq) in bodies of *Dendrobaena alpine* during experiment.

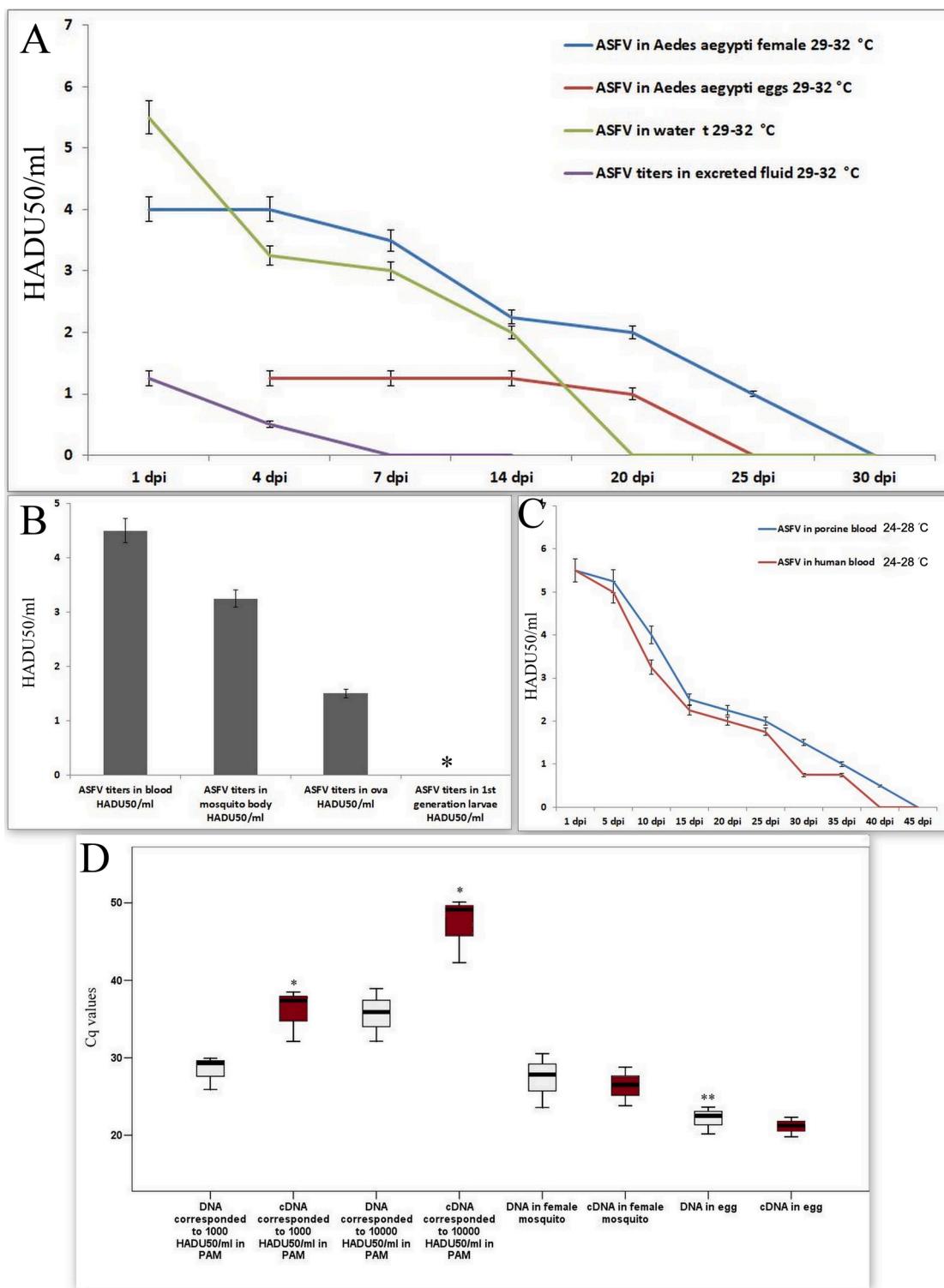


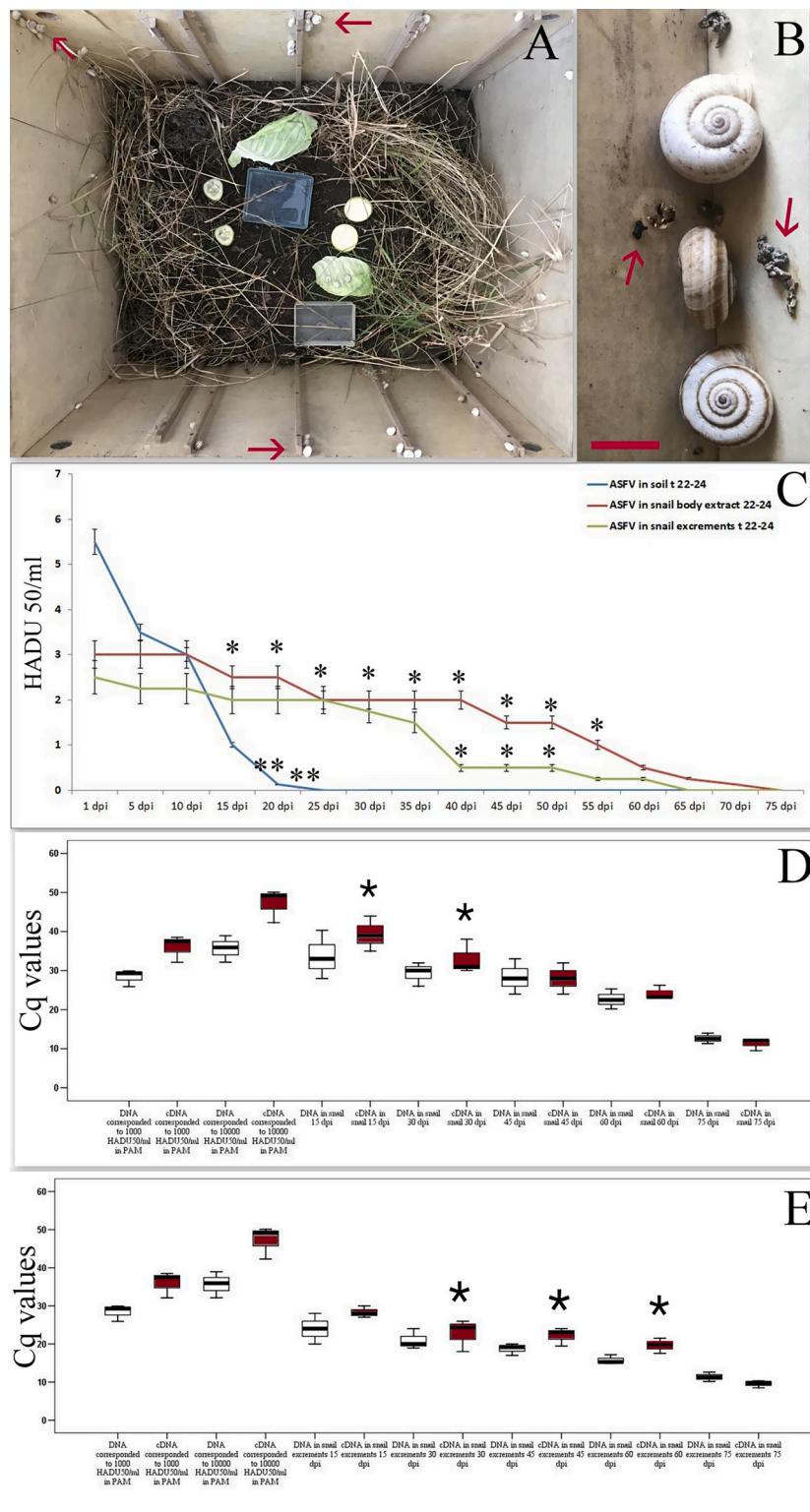
Fig. 4. ASFV titers Aedes bodies, eggs, and in environment. A. Decreased levels of ASF virus in female mosquito bodies, in eggs of *A. aegypti*, in environmental water infected by ASFV without mosquitoes, and in excreted fluid after infection via blood feeding in units of hemadsorption (Ig). B. Levels of ASFV (Armenia08) in blood used for feeding, in bodies on the 10th day after infection and eggs on the 1 st day after oviposition of *A. aegypti* expressed in units of hemadsorption (Ig). C. ASFV surviving in human and porcine blood at temperature 24–28 °C. D. Levels of transcription of ASFV K196R gene (expressed in Cq) in bodies of blood-fed female mosquitoes on 10th day after infection and eggs on 1 st day after oviposition obtained by qRT-PCR data.
* Significant compared to DNA levels ($p < 0.05$ by Mann-Whitney U test).

soil. Our data showed that the survival of the ASF virus in the presence of earthworms infected by injection does not differ from the survival time of the virus in the soil that does not contain earthworms.

Analysis of transcription of viral genes using qReal-TimePCR

(thymidine kinase K196R) did not reveal transcriptional activity either in the infected soil or in earthworms (Fig. 3D).

The study of a new generation of earthworms (a new generation was born 30–50 days after the start of the experiment) did not reveal the



presence of a virus. The search for the virus was carried out by the method of hemadsorption and qReal-TimePCR. The presence of transcriptional activity of ASF genes was not shown either.

3.3. Experimental horizontal and vertical transmission of African swine fever Virus by *Aedes aegypti*

Fig. 4A shows that the number of hemadsorbing units of the virus in

the female mosquitoes after feeding is lower (0.5 lg) than the amount of the virus in the blood from the feeder (however this difference was insignificant; Mann-Whitney U: $P > 0.05$). It indicates the entry of the virus into the mosquito's body.

We investigated females prior to laying eggs on days 1 and 4, and females who laid eggs on 7 and 10 days post-feeding. ASFV was detected in all blood-fed mosquitoes and most eggs (27 of the 30 eggs tested). The amount of the virus in the blood used to feed mosquitoes and the amount

of the virus in the body of the female mosquitoes after feeding do not differ significantly (Mann-Whitney U: $P > 0.05$). Comparison of virus levels in the water environment (without mosquitoes) and the body of female *Aedes* reveals a slower inactivation of the virus in the mosquito. However, these differences are not significant (Mann-Whitney U: $P > 0.05$). It's well-known that mosquitoes concentrate blood they ingest and excrete excess fluid anally; we also investigated this excreted fluid (Fig. 4A), and compare with survival time of the ASF virus in water. Thus total survival time of the ASF virus in the body of mosquitoes (in imago and eggs) was about 50–60 days - 35–40 days in the body of an imago and 20–25 days in eggs. Comparison of virus levels in infected blood, the bodies of female mosquitoes (10 days after infection), eggs immediately after oviposition, and larvae of the 1 st generation are shown in Fig. 4B. We did not detect any infectious virus particles in the new generation of mosquitoes when studied by the hemadsorption method.

The virus can also persist in blood swallowed by mosquitoes. Therefore, we present data on the survival of the ASF virus in the blood of humans and pigs. As follows from Fig. 4C, the virus is able to survive at a temperature of 24–28 °C for about 1 month or few more (up to 35–40 days), while there was no significant difference between survival in human and porcine blood.

We also studied the transcriptional activity of several ASF genes (data are given only for K196R); however, we did not find any evidence of viral transcription in the body and eggs of mosquitoes (Fig. 4D).

Then we investigated the possible contamination of pigs by oral contamination with ASFV-infected mosquitoes. Despite the 15-day observation of pigs that received three bodies of female mosquitoes which received blood 15 days earlier, we have not identified cases of pig infection with the virus. Investigations of the next generation of larvae detected only trace amounts of the ASFV genomes (by qReal-TimePCR) and absence of infection particles (measured by HADU₅₀/mL). Our data indicate that the natural transmission of the ASF virus through the oral administration of mosquitoes to pigs has a low likelihood and may only be possible through high amounts of ingested insects.

3.4. Stability and recovery of ASFV from land snails (*Xeropicta*)

Xeropicta is a genus of small to medium-sized, air-breathing land snails common to Eurasian regions. For experimental purpose 100 snails were transferred into separate boxes with a soil with grass. The boxes were sealed with plastic net to prevent the escape of snails during the experiment and to ensure a stable water saturated atmosphere. This type of cultivation makes it easier to collect biological samples. (Fig. 5A; B). Infectious titers of ASFV virus in *Xeropicta derbentina* body and feces were significantly lower than virus levels in soil and water since the 1st day of the experiment. However, the rate of decline in ASF virus levels in soil was much higher than in molluscs. By 15 dpi, the difference in virus levels in soil and snails was significant (Fig. 5C, Mann-Whitney U: $P < 0.01$). At 20 dpi, a significant portion of the soil samples was negative for the virus content (with the HADU₅₀/mL test). ASFV infectious particles from snail specimens and their feces persisted about three times longer than soil samples (Fig. 5C, Mann-Whitney U: $P < 0.01$). The virus level in snail feces was lower than in *Xeropicta derbentina* bodies by about 1–1.5 log HADU₅₀/mL. This difference was observed in all experimental samples. Long-term isolation of the virus from snail feces suggests that the virus is likely to remain in the gastrointestinal tract of *Xeropicta*. We have shown that virus in the body of *Xeropicta derbentina* remains detectable for longer compared to soil or water. It should be noted that qReal-TimePCR revealed the presence of the gene (K196R) for longer (Fig. 5D). It seems important that in all samples isolated from the bodies of snails or from faces (Fig. 5E), the transcriptional activity of viral genes was shown (shown by the example of a gene K196R). Nevertheless, we did not find an increase in the infectious titers of the virus in investigated samples, although the transcriptional activity of viral genes was constantly demonstrated until the disappearance of the virus (Fig. 5 D, E).

3.5. Transmission of African swine fever virus in the process of natural feeding

Three pigs were fed with a diet containing bodies of ASFV infected female mosquitoes (described previously). Each pig received three bodies of female mosquitoes which received infected blood 15 days earlier. Pigs were maintained 15 days and do not express any symptoms of disease. Presence of the virus also detected in blood by HADU₅₀/mL. We took mosquitoes with contaminated blood by 15 days after infection, based on the fact that mosquitoes survive for about 10–20 days after laying their eggs. The consumption of live mosquitoes by pigs is very unlikely.

Another three pigs (Large White breed) were fed with a diet containing bodies of ASFV infected *Xeropicta derbentina* (described previously). Each pig received three bodies of *Xeropicta derbentina* which fed on contaminated water 45 days earlier and live in contaminated soil. Pigs were maintained 15 days and do not express any symptoms of disease.

4. Discussion

Today, the ASF virus has ceased to be an African endemic and has become a part of the ecosystem of Eurasia. At the same time, it should be noted that there is no specific vector in the current Eurasian ecosystem, and so far no reservoir has been identified that can sustain replication and release of the virus into the environment in the long-term, since the boar cannot withstand the cycle of endemic infection, due to high lethality and low contagiousness (Carlson et al., 2020).

One of the most interesting features of the ASF virus is its ability to persist for a long time in regions without virus-specific reservoirs. Earlier it was shown (Arzumanyan et al., 2021) that the virus can't persist in the carcasses and bones/bone morrows of dead pigs for more than several months and does not appear to remain infectious after several weeks. These data, in general, coincide with the information (Zani et al., 2020) on the persistence of the infectious ASF virus in the bodies of wild boars. The data given in the article (Fischer et al., 2020) indicate a slightly longer period of preservation of the infectious virus in the corpses of pigs, but this is most likely due to the temperature of the virus in the samples of pig tissue. However, regardless of the survival time in pig carcasses, the virus continues to persist in the Eurasian region and cause occasional outbreaks of the disease, which suggests natural circulation.

Generally, transmission of viruses occurs by a direct transmission during different types of contact between two hosts, or by an indirect transmission through the environment. Several factors, both abiotic and biotic, determine the persistence of these viruses released in the environment, which can last from a few seconds to several years (Labadie et al., 2020). According to our data obtained as a result of the analysis of the safety of the virus in bone marrow samples (Arzumanyan et al., 2021; Niederwerder et al., 2019), the minimum dose at which the ASFV can infect pigs is about 1 HADU₅₀/mL. At titers below this level, successful infection of animals is unlikely, but this will depend there on the amount of contaminated material and route of infection.

There is evidence that the soil contaminated with the ASF virus, on which the boar carcass was decomposed, might be one of the factors contributing to the circulation of the virus in the new ecosystem (Probst et al., 2017). Our previous data (Arzumanyan et al., 2021) suggest that this is unlikely, especially at temperatures of 20 °C and above. Abiotic environmental factors such as high temperatures and UV radiation usually have negative effects on virus survival and dispersal in ecosystems. In addition, our data revealed a slightly longer existence of infectious particles of the ASF virus (about 20–25%) in the soil compared to the data by (Carlson et al., 2020). This is probably the result of a slightly lower temperature in our experiments.

Therefore, we investigated the possible biological factors affecting the survival of the ASFV in the environment.

Experimental studies carried out with earthworms (*Dendrobaena*

alpina) and the ASF virus suggest that earthworms do not contribute to its maintenance in the environment, since co-cultivation did not change the survival time of the virus in the soil.

We co-incubated ASFV with destroyed *Paramecium* cells to reveal the role of enzymes derived from destroyed ciliate in the inactivation of ASFV and found that enzymes in solution have no role in the inactivation. Ciliated grazers (*P. caudatum* in our case) could play a role in the inactivation of viruses, either through the production of metabolites that adversely affect the virus particles, or by direct use of the viruses as a nutrient source. However, the true mechanism involved in the inactivation of ASFV by *P. caudatum* cells is unknown. One of the conclusions of our research is that the presence of ASFV impacts on the population of *P. caudatum* leading to a sharp increase of their number in the medium. The growth of the number of ciliate when the virus was present in water environment can be explained by the fact that viruses serve as a nutrient source for *P. caudatum*.

Our studies have shown that ASFV infectious particles from *Xeropicta derbentina* snails and their feces persisted for 60 dpi; much longer than soil samples. However, this data alone does not provide sufficient evidence for demonstrating the possibility of ASF virus replication in the *Xeropicta derbentina* organism. Increased transcriptional activity of gene K196R is not accompanied by an increase in infectious virus titers, determined by the HADU₅₀/mL test. However, the transcriptional activity of viral genes may indicate favorable conditions for the relatively long survival of the virus and/or the possible low level replication of the virus that can partially compensate for the natural decay of the virus.

In this study, we also investigated the possibility of preserving and transmitting the ASF virus through *Aedes* mosquitoes - in other words, the characteristics of *A. aegypti* as a vector for the ASF virus. The use of human blood instead of pig blood was caused by the low efficiency of feeding *Aedes* with artificial feeding. This is due to the taste preferences of mosquitoes as they very difficult began feed porcine blood (Pon-lawat, Harrington, 2005). Within this study we tested 1. Vertical transmission of virus from mother to eggs/offspring, 2. Horizontal transmission from female mosquitoes to pig through consumption. The absence of viral transcription and a decrease in the amount of the virus in mosquitoes indicate that ASFV does not replicate in *A. aegypti* mosquitoes.

According to Luhong (2018), one of the main criteria for viral vectors is the isolation of virus from carriers. This criterion related to mosquitoes in natural conditions yielded negative results. However, in experimental conditions virus was successfully isolated from the female mosquitoes. Also, we can conclude that under experimental conditions, mosquitoes can be infected with ASFV after feeding on the blood of the viral host, in agreement with the second criterion of viral vectors. The third criterion about the possibility of transmitting the virus to the vertebrate host during the experiment gave a negative result. The main reason is that ASFV positive mosquitoes (who received the virus with blood) did not carry the live virus long enough in a sufficient amount to transfer it to pigs. In conclusion, we find no evidence for vertical transmission of ASFV by *Ae. aegypti* and no evidence that the virus can be transferred to pigs through ingestion. Based on our findings, mosquitoes are unlikely to play a role in ASFV transmission in nature.

Thus, in experimental studies on phylogenetically distant organisms, we have shown their different variants of influence on the ecology of the ASF virus. The influence of the earthworm (*Dendrobaena alpina*) on the ecology of the ASF virus is absent it is only possible that the virus will not spread very much when the earthworm moves in soil. Ciliates (*Paramecium caudatum*) significantly increase the rate of ASF virus disappearance from the aquatic environment, probably using the virus as a food source. Mosquitoes (*Aedes aegypti*) do not provide significant support for the persistence of ASF virus in the environment, however are likely to contribute to the spread, but not transmission of the virus. In land snails (*Xeropicta derbentina*), the ASF virus lasts much longer than in the soil. Moreover, the snails were able to maintain the transcription of viral genes, although the question of full-fledged viral replication is still

open. In addition, taking into account the active movements of snails, we can also confirm their role in the spread, but not transmission of the virus. The virus is likely to be localized in the intestines of snails as it is regularly excreted from their feces.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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