No evidence of African swine fever virus replication in hard ticks

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Abstract

African swine fever (ASF) is caused by African swine fever virus (ASFV), a tick-borne DNA virus. Soft ticks of the genus *Ornithodoros* are the only biological vectors of ASFV recognized so far. Although other hard ticks have been tested for vector competence, 2 commonly found tick species in Europe, *Ixodes ricinus* and *Dermacentor reticulatus*, have not been assessed for their vector competence for ASFV. In this study, we aimed to determine whether virus replication can occur in any of these 2 hard tick species (*I. ricinus* and/or *D. reticulatus*), in comparison with *O. moubata* (the confirmed vector), after feeding them blood containing different ASFV isolates using an improved in vitro system. DNA quantities of ASFV in these infected hard ticks were measured systematically, for 6 weeks in *I. ricinus*, and up to 8 weeks in *D. reticulatus*, and the results were compared to those obtained from *O. moubata*. There was evidence of virus replication in the *O. moubata* ticks. However, there was no evidence of virus replication in *I. ricinus* or *D. reticulatus*, even though viral DNA could be detected for up to 8 weeks after feeding in some cases. This study presents the first results on the possible vector competence of European hard (ixodid) ticks for ASFV, in a validated in vitro feeding setup. In conclusion, given the lack of evidence for virus replication under in vitro conditions, *D. reticulatus* and *I. ricinus* are unlikely to be relevant biological vectors of ASFV.

Keywords: African swine fever virus; In vitro feeding; *Ixodes ricinus*; *Dermacentor reticulatus*

Introduction

African swine fever (ASF) is a highly contagious haemorrhagic disease of swine, caused by African swine fever virus (ASFV), an enveloped double-stranded DNA virus from the family Asfarviridae, genus Asfivirus. Infection usually results in high morbidity and mortality (Costard et al., 2012). Since it was first described (1921), African swine fever has been present in most of sub-Saharan Africa, with most of the incidental introductions into Europe and the Americas eventually resulting in eradication (with the exception of Sardinia). However, after the 2007 ASFV outbreak in Georgia (Rowlands et al., 2008), the disease continued to spread, reaching European neighbouring countries. At the time of writing, ASFV is still circulating in Russia (OIE, 2013), mostly between wild boar and free-ranging domestic pigs (Gogin et al., 2013).

ASFV is a tick-borne virus (Labuda and Nuttall, 2004), and soft ticks (*Ornithodoros* spp.) have been identified as vectors of ASFV. In Africa, an intricate cycle between warthogs (*Phacochoerus africanus*), domestic swine, and *Ornithodoros* ticks (particularly *O. moubata*), is relevant in the maintenance of an endemic infection (Plowright et al., 1994; Thomson, 1985). On the Iberian Peninsula, *Ornithodoros erraticus* has also been associated with disease reoccurrence in a sporadic ASF outbreak in Portugal in 1999 (Boinas et al., 2011). Upon ingestion of blood containing ASFV, ticks may develop a persistent infection, with high virus titres in a number of tissues and organs, both in the *O. moubata/porcinus* complex (Greig, 1972; Kleiboeker et al., 1998; Plowright et al., 1970a, 1970b) and in *O. erraticus* ticks (Basto et al., 2006; Endris and Hess, 1992; Endris et al., 1992).

Moreover, ASFV-infected *Ornithodoros sonrai* ticks (Vial et al., 2007) have been found in the field, and in vitro studies have suggested that several other *Ornithodoros* species such as *O. savignyi* (Mellor and Wilkinson, 1985), *O. puertoricensis* (Hess et al., 1987), *O. turicata* (Hess et al., 1987), *O. coriaceus* (Groocock et al., 1980) can also act as vectors of ASFV. However, none of the latter ticks has yet been confirmed as vector for the transmission of ASFV in the field.

ASFV vector competence of hard ticks (Ixodidae) has been assessed on *Rhipicephalus* spp. (Sanchez Botija, 1963), *Rhipicephalus simus* (Plowright, 1977; Plowright et al., 1994), *Rhipicephalus bursa* (Kovalenko et al., 1967; Plowright et al., 1994), *Amblyomma variegatum* (Plowright, 1977), *Hyalomma* spp. (Plowright et al., 1994), *Amblyomma americanum* and *Amblyomma cajennense* (Groocock et al., 1980) either by experimental infection or by field collection to determine the presence of ASFV. Field specimens were negative for ASFV, and although ASFV could be detected in *R. simus* nymphs (Plowright et al., 1994) for up to 5–6 weeks, and in both *A. americanum* and *A. cajennense* for 4–7 days after a viraemic blood meal, no hard ticks transmitted ASFV to susceptible pigs after experimental infection. Given that some hard ticks may carry ASFV for some time, it is possible that they may act as mechanical vectors; similar to the European stable fly, *Stomoxys calcitrans*, which has been shown to mechanically transmit ASFV to pigs up to 24 h post-infective meal (Mellor et al., 1987).

Of the hard ticks studied so far, only *Hyalomma* spp. and *Rhipicephalus bursa* are present in Europe (Estrada-Peña et al., 2012). However, no studies have been published addressing replication or maintenance of ASFV in other ticks commonly reported in Europe, such as *Ixodes ricinus* (Medlock et al., 2013) and *Dermacentor reticulatus* (Estrada-Peña et al., 2012).

Both tick species are known to be involved in the epidemiology of other tick-borne viruses; i.e. with *I. ricinus* being a vector of tick-borne encephalitis virus (TBEV) and *D. reticulatus* being a vector of Omsk haemorrhagic fever virus (OHFV) (Jongejan and Uilenberg, 2004; Labuda and Nuttall, 2004).

However, feeding habits differ between the 2 tick species. *Ixodes ricinus* ticks feed on a wide range of hosts, including domestic and wild pigs (Farkas et al., 2013). In contrast, *D. reticulatus* host preference strongly depends on life stage, with adults feeding on larger mammals and nymphs mostly on small mammals (Farkas et al., 2013). Both tick species could be involved in ASFV transmission either via mechanical transmission by interrupted feeding (by adult males) or via biological transmission, either transovarially (via ASFV-infected females) or transstadially (via ASFV-infected *I. ricinus* nymphs).

In this study, *I. ricinus*, *D. reticulatus*, and *O. moubata* (one of the confirmed vector species) were fed in vitro with blood containing different ASFV isolates. DNA quantities of ASFV in these hard ticks were measured systematically, for 6 weeks in *I. ricinus* and up to 8 weeks in *D. reticulatus*, and the results were compared to those found in *O. moubata*. The purpose of this comparison was to examine if replication can occur in both species of hard ticks. Such knowledge is relevant to better understand the possible role these hard ticks could play as biological or mechanical vectors of ASFV.

Materials and methods

Ticks

Ixodes ricinus and *D. reticulatus* ticks originated from the Netherlands, whereas *O. moubata* was obtained from a laboratory colony maintained in Israel. Developing stages of both hard tick species were maintained at the Utrecht Centre for Tick-borne Diseases (UCTD) at 23°C and 85% relative humidity, created by a saturated potassium chloride solution, and a 12-hour photoperiod. The experiments were carried out in late spring and summer. Ticks were tested by PCR and reverse line blot hybridization for pathogens prior to the start of the experiments (Gubbels et al., 1999). Specific pathogen-free, laboratory-reared *I. ricinus* nymphs (n=3360), *D. reticulatus* adults (n=720, 1:1 male to female ratio), age between 3-6 months post moulting and *O. moubata* nymphs of different development instars, namely the third nymphal instar (N3, n=300) and fourth nymphal instar (N4, n=300) were used. Between feeding and sample collections, hard ticks were maintained at 20–24°C, with a relative humidity (RH) of 85%. *Ornithodoros moubata* ticks were maintained at 27–28°C, with a RH of 85–90%.

Virus

Six ASFV isolates were used in our study. Two were originally isolated from ticks in Portugal (OURT 88/1) and in Zambia (LIV 13/33), respectively, and 4 were isolated from infected pigs: Georgia 2007/1, Malta'78, Netherlands'86, Brazil'78. Virus stocks were obtained from previous animal experiments, either by collecting defibrinated blood or by preparing spleen homogenates from infected pigs (Table 1).

Preparation of blood for in vitro feeding

Pig blood was collected weekly from a local slaughterhouse. Immediately after collection, the blood was stirred rapidly with a glass pipette for 15–30 min, and the fibrin clot that attached to the glass pipette was removed. The defibrinated pig blood was stored at 4°C. During acquisition feeding, the defibrinated pig blood was spiked daily with the 6 different virus suspensions, resulting in a 1:10 dilution of the original ASFV titres.

In vitro feeding units

The improved in vitro system used in this study, recently described by Fourie et al. (2013), results from several adaptations of a system described previously (Kröber and Guerin, 2007a, 2007b). In our system, silicone membranes were impregnated with cattle odour immediately before being fixed to the bottom of hollow plastic tubes, with a diameter of approximately 3.5 cm. Ticks were placed inside the feeding unit, on top of the feeding membrane, and forced to remain near the membrane of the feeding unit by a movable plastic lid that permitted air circulation. A volume of 3.1 ml of pig blood per well was pipetted into 6-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany), and feeding units were fitted into these wells.

Acquisition feeding of *O. moubata* (soft ticks)

Each acquisition feeding consisted of 12 feeding units (as described above), one for each combination of ASFV isolate with each *O. moubata* nymphal instar (N3 or N4). Each feeding unit contained approximately 50 *O. moubata* nymphs. Plates with feeding units, ticks, and ASFV spiked blood were placed on a heated plate (37°C). The nymphs were allowed to engorge completely over a period of maximum 5 h. After each moult, *O. moubata* ticks were re-fed using the same system described previously, but using uninfected pig blood.

Acquisition feeding of *I. ricinus* and *D. reticulatus* (hard ticks)

Acquisition feedings were repeated thrice for both hard tick species. Each acquisition feeding consisted of 24 feeding units, 4 for each ASFV isolate. Each feeding unit/well contained either 40–50 *I. ricinus* nymphs or approximately 10 *D. reticulatus* adults (5 females, 5 males) feeding on a membrane. Differences in numbers of ticks per unit were due to differences in tick size.

During the period of (pre) attachment, the hard ticks were initially placed on uninfected defribinated blood, as not to waste viral infective material. As soon as the first ticks started to feed, the uninfected blood was replaced by blood spiked with ASFV. Blood in the wells was changed approximately every 12 h, for 7 days. Plates with feeding units, ticks, and infected blood were placed in a water bath at 37°C, in the dark, with RH >70%.

Sample collection

Ten unfed *O. moubata* nymphs (5x N3, 5x N4), 10 unfed nymphal *I. ricinus*, and 10 unfed adult *D. reticulatus* ticks were sampled before acquisition feeding and tested as one pool sample per species to assure that the used ticks were free of ASFV. The first *I. ricinus* samples were taken for investigation immediately after acquisition feeding and then every week up to 6 weeks after feeding. The engorged nymphs did not complete the moulting process within the observational period of 6 weeks under the prevailing experimental conditions. The number of ticks sampled is shown in Table 2. *Dermacentor reticulatus* ticks were sampled and investigated one week after acquisition feeding and then every week up to 8 weeks after feeding (Table 2). Sampling immediately after feeding was skipped because of the limited number of ticks available and to maximize the length of period of sampling after feeding. *O. moubata* ticks were sampled immediately after acquisition feeding, one week after feeding, one week after the first moult (varying from 2-4 weeks after feeding, depending on the instar and the virus isolates) and 7 weeks after the acquisition feeding (those that had moulted to adults had received a second feeding [with uninfected blood], so this corresponded to one week after first oviposition in adults that were fed ASFV containing blood as N4 nymphs) (Table 2). This slightly different sampling schedule was due to the interference of moulting and in-between additional feedings with a weekly sampling schedule.

Processing of ticks

Ticks were rinsed in ethanol prior to placing each individual tick in a 2.0-ml PCR clean Eppendorf Safe-Lock Tube (Eppendorf, Nijmegen, The Netherlands) with 100 µl of PBS (Gibco, Invitrogen, Breda, The Netherlands) and a 5-mm diameter stainless steel ball (QIAGEN Benelux, Venlo, The Netherlands). Samples were then immediately frozen at –70°C for at least 10 min. While still frozen, ticks were homogenized by shaking for 5 min at 50-Hz frequency, using a TissueLyser LT (QIAGEN Benelux, Venlo, The Netherlands). Another 900 µl of PBS was added and the sample was centrifuged for 2 min at $9500 \times$ g. Supernatants were transferred to clean tubes (Micronic Europe, Lelystad, The Netherlands) and stored at –70°C until they were analysed by quantitative real-time polymerase chain reaction (qPCR).

Quantitative real-time polymerase chain reaction (qPCR)

Samples were analysed in a qPCR to determine the concentration of viral DNA, according to the procedure described in de Carvalho Ferreira et al. (2012). Standard curves for each ASFV isolate used (OURT 88/1, LIV 13/33, Georgia 2007/1, Malta'78, Netherlands'86, Brazil'78) were prepared by adding different dilutions of virus stock to ASFV-negative tick suspensions. For each curve, 4 dilutions were used, with titres of 10^5, 10^3, 10^2, 10^1 $TCID_{50}$/ml. The viral DNA concentration of each individual sample was then calculated using the qPCR run-specific standard curve, based on the cycle threshold (CT) values. For tick samples, it was expressed as median tissue culture infective dose equivalents ($TCID_{50}$ eq.) per tick. $TCID_{50}$ equivalents do not necessarily represent live virus, but represent a relative measure of the amount of viral DNA present in a sample.

Statistical analysis

The considerable number of ticks that tested negative in qPCR made it invalid to use a statistical method assuming a normal distribution of the data. For that reason, data were analysed with a so-called hurdle model (Zuur et al., 2009). In order to use this count data regression model, the ASFV DNA titres (in $\log_{10}TCID_{50}$ eq.) had to be transformed to a discrete count variable, which was done in 3 steps.

(i) A linear regression analysis was made across qPCR runs, relating all CT values measured with all dilutions of the standard curve, thus generating a mean regression line with its

corresponding slope and intercept. (ii) A normalized CT was calculated from the ASFV DNA titres, using the mean slope and intercept parameters, by applying the formula:

Normalized CT = (ASFV DNA titres – intercept) / slope; with slope = –0.258 and intercept = 9.02

(iii) The normalized CT was then converted into a score variable using the following formula:

Score= (maximum normalized CT - normalized CT) + 1

Values exceeding the maximum normalized CT (44) were considered negative and given the value 0. The CT value of 44 was transformed to score 1, of 43 to score 2 etc. Next, the probability that a tick tested ASFV-positive in qPCR (score 0 or score $\geq$1) was analysed by a logistic regression model using a binomial distribution with a logit link function. Subsequently, the non-negative results (score $\geq$1) were analysed by a regression model using a Poisson distribution, with a log link function, and the natural logarithm of the maximum score (30) was used as offset variable.

In both parts of the hurdle model, the variables time (weeks), virus isolate (6 different ASFV isolates used to infect ticks), and tick species (3 different species), and all their possible statistical interactions were used to predict the outcome of interest (score 0 or score $\geq$1 in the first part of the hurdle model; or score in the second part of the model).

Model selection was based on Akaike's Information Criteria (AIC). Statistical analyses were performed with R, version 2.11.1 (R Development Core Team, 2008) using the package "pscl" (Zeileis et al., 2008). Statistical significance was considered when $p<0.05$.

Results

O. moubata

Immediately after feeding the *O. moubata* ticks, ASFV titres in the range of approximately $10^{3.0}$–$10^{4.5}$ TCID$_{50}$ eq./tick were observed in individual ticks (Figs. 1 and 2, week 0). Highest ASFV titres were observed in the Georgia 2007/1 and Malta'78 groups. This was consistent with ASFV titres in the blood used to feed the ticks, which were also highest for these 2 ASFV isolates (Table 1). Variation within the groups was limited (from 0.2 log TCID$_{50}$ eq./tick between the highest and lowest titre in the Brazil'78 group, to 1.1 log TCID$_{50}$ eq./tick in the Georgia 2007/1 group), indicating that the ticks ingested similar amounts of blood. Only one of the ticks, in the OURT88/1 group, was PCR-negative on the first sampling (Table 2).

After an initial decrease of median ASFV titres, an increase was observed from week 3 or 4 after the acquisition feeding in some of the groups (Figs. 1 and 2). Virus titres in individual ticks, higher than the titres at day 0, were considered indicative for virus replication. This was observed in several ticks in the OURT88/1, LIV13/33, and Netherlands'86 groups, with some individual cases in the Malta'78 (4 weeks) and Brazil'78 (7 weeks) groups (Figs. 1 and 2). At the same time, the variation also increased, indicating that virus replication took place in only a proportion of the ticks, while in other ticks the virus was decreasing or completely disappeared. The LIV13/33 group was the only group showing a significant overall increase of virus titres in time (Table 3). All the other groups showed an overall decline (although not always significantly below 1) (Table 3). Furthermore, the odds of obtaining negative PCR results were significantly higher in the period of 3–7 weeks, compared to the first 2 weeks after feeding. These findings are consistent with the fact that high ASFV titres, which indicate virus replication, were only found in some of the ticks in each group and that also an increasing number of ticks became PCR-negative (22 out 30 ticks at week 7, Table 2). In the Georgia 2007/1 group, on the other hand, only decreasing titres were observed (Fig. 2), together with an overall significant decrease of virus titres in time (Table 3) indicating that there was no virus replication at all in *O. moubata*.

I. ricinus

Immediately after feeding the *I. ricinus* ticks, ASFV titres were lower than in the *O. moubata* ticks (ranging from approximately $10^{-0.3}$ to $10^{3.8}$ TCID$_{50}$ eq./tick) (Figs. 1 and 2, week 0). Except for the Brazil'78 group, these differences were statistically significant (Table 3). Variation in ASFV titres of *I. ricinus* (up to 4.1 log TCID$_{50}$ eq. /tick between the highest and lowest titre; Figs. 1 and 2) was much larger than for *O. moubata*, indicating that there were large differences between individual ticks in ingested blood containing ASFV. Nevertheless, only 2 ticks (one in the Malta'78 group and one in the Netherlands'86 group) were PCR-negative after feeding (Table 2).

An overall decrease of virus titres in the ticks was observed in time (Table 3). Contrary to *O. moubata*, no high virus titres developed in ticks within the time they were followed (Figs. 1 and 2). The overall decrease of virus titres was statistically significant for all 6 viruses (Table 3). Furthermore, the odds of obtaining negative PCR results were significantly higher in the period of 3–6 weeks, compared to the first 2 weeks after feeding. This indicates that none of the ASFV isolates replicated in *I. ricinus* ticks up to 6 weeks after feeding.

D. reticulatus

One week after feeding the *D. reticulatus* ticks, virus titres were even lower than in the *I. ricinus* ticks (ranging from approximately $10^{-0.5}$ to $10^{1.7}$ TCID$_{50}$ eq./tick) (Figs. 1 and 2, week 1). When extrapolated to expected virus titres immediately after feeding, these differences were statistically significant, except for the Brazil'78 group (Table 3). Variation in ASFV titres of *D. reticulatus* ticks (up to 2.3 log TCID$_{50}$ eq./tick between the highest and lowest titre) was much larger than for *O. moubata*, but smaller than for *I. ricinus*. Furthermore, 8 out of 17 *D. reticulatus* were PCR-negative one week after feeding (Table 2). This indicates large differences in ingested blood containing ASFV and an overall limited blood intake of the ticks. In time, an increase of PCR-negative ticks was observed, with the odds of obtaining negative PCR results in the period of 3–8 weeks significantly higher than in the first 2 weeks after feeding. Virus titres in the remaining PCR-positive ticks showed no significant decrease or increase, except for the Netherlands'86 group. However, the increase in time observed in this group was driven by a single tick showing a relatively high ASFV titre (1.75 log TCID$_{50}$ eq.) at the end of the sampling period (8 weeks after feeding). Yet, this ASFV titre was still considerably lower (over 3 log TCID$_{50}$ eq./tick) than ASFV titres on *O. moubata* ticks in which virus replication was apparent (Figs. 1 and 2). In contrast to observations in *O. moubata*, no high ASFV titres developed in *D. reticulatus* within the time they were investigated (Fig. 2). This indicates that none of the ASFV isolates replicated in *D. reticulatus* up to 8 weeks after feeding.

Discussion

In this study, ASFV replication was confirmed by DNA quantification in *O. moubata* with several ASFV isolates. In contrast, there was no evidence of virus replication in *I. ricinus* or in *D. reticulatus*, even though viral DNA could be detected for up to 6 and 8 weeks after feeding, respectively, in some cases.

ASFV replication was not observed in all *O. moubata* ticks. Differences in infection rate, infectious dose, and ASFV replication found in *O. moubata* have been previously reported to depend on the ASFV isolate (Kleiboeker et al., 1999; Plowright et al., 1970b), in which the presence of specific multigene families (MGF) 360 and 530 has been associated with the replication in ticks (Burrage et al., 2004). For that reason, multiple virus isolates were used in the experiment. Virus replication in ticks has also been shown to depend on the geographical

origin of tick batches (Greig, 1972; Plowright et al., 1970b), which is linked to the identification of geographical sublineages of soft ticks in Africa (Bastos et al., 2009). This could also explain why no replication of the Georgia 2007/1 virus isolate was observed in *O. moubata*, even though this virus did replicate in *O. erraticus* ticks (Diaz et al., 2012). Given that the Georgia 2007/1 isolate belongs to genotype II, which includes other contemporary isolates circulating in Mozambique, Madagascar, and Zambia (Rowlands et al., 2008), the existence of a specific lineage of *O. moubata* circulating in these regions that supports the replication of Georgia 2007/1 isolate cannot be disregarded.

There were marked differences in ASFV titres immediately after feeding, which can be related to a variation in the ingested amounts of blood. This variation is partly due to differences in size of the ticks, but is probably also caused by different feeding habits and suitability of the feeding system for each of the used tick species. *Ornithodoros moubata* ticks are fast feeders, engorging up to 5–10 times their initial body weight within a few minutes to hours, with females feeding and depositing eggs repeatedly, and with several nymphal instars (Sonenshine, 1991). Using our in vitro system, all soft ticks became fully engorged and moulted within the expected period of time (2–3 weeks). Hard ticks, on the other hand, are slow feeders, taking several days or even weeks to fully engorge, ingesting most of their blood meal within the last 24–48 h before detachment, with females depositing eggs only once, and with only one nymphal instar (Sonenshine, 1991). Preliminary experiments (data not shown) feeding *D. reticulatus* nymphs in vitro presented unsatisfactory results, whereas for adult ticks results were acceptable. Still, in our experiments, roughly 20% of the hard ticks were successfully fed, meaning they acquired ASFV in sufficient quantities, on the membranes within the feeding time frame. Despite the successful feeding of *I. ricinus* ticks, preliminary experiments showed (data not shown) that ticks did not moult at the expected time after feeding (approximately 6–7 weeks). Thus, further optimization of the feeding system, including the conditions under which the ticks are kept subsequently, may be needed to increase both the percentage of successful feedings and the moulting rate. Nevertheless, sufficient numbers of engorged ticks were available for the experiment, and PCR results after feeding confirmed the uptake of virus. Hard ticks were tested for up to 6–8 weeks after feeding, which was sufficient for the soft ticks to show evidence of virus replication.

Even though no evidence of ASFV replication was found in *I. ricinus* and *D. reticulatus*, several of these ticks were still PCR-positive by the end of the study period. Decreasing levels of viral DNA in time suggest that both inactivation of the virus and decay of the viral DNA

are taking place. And on the other hand, based on PCR data, there was reliable evidence of virus replication in *O. moubata*, which contrasted with the comparative lack of replication in hard ticks. No virus isolation was attempted to confirm or rule out the presence of viable virus, and thus the precise moment when infectious ASFV may have been cleared from the hard ticks could not be determined.

In conclusion, this study presents the first results on a possible vector competence of European hard (ixodid) ticks for ASFV in an in vitro feeding setup. There was no evidence of virus replication *D. reticulatus* and *I. ricinus*, making both of them unlikely as biological vectors of ASFV.

References

Basto, A. P., Nix, R. J., Boinas, F., Mendes, S., Silva, M. J., Cartaxeiro, C., Portugal, R. S., Leitao, A., Dixon, L. K., Martins, C., 2006. Kinetics of African swine fever virus infection in *Ornithodoros erraticus* ticks. J. Gen. Virol.. 87**,** 1863-71.

Bastos, A. D., Arnot, L. F., Jacquier, M. D., Maree, S., 2009. A host species-informative internal control for molecular assessment of African swine fever virus infection rates in the African sylvatic cycle *Ornithodoros* vector. Med. Vet. Entomol. 23**,** 399-409.

Boinas, F. S., Hutchings, G. H., Dixon, L. K., Wilkinson, P. J., 2004. Characterization of pathogenic and non-pathogenic African swine fever virus isolates from *Ornithodoros erraticus* inhabiting pig premises in Portugal. J. Gen. Virol.. 85**,** 2177-2187.

Boinas, F. S., Wilson, A. J., Hutchings, G. H., Martins, C., Dixon, L. J., 2011. The persistence of African swine fever virus in field-infected *Ornithodoros erraticus* during the ASF endemic period in Portugal. PloS ONE. 6**,** e20383.

Burrage, T. G., Lu, Z., Neilan, J. G., Rock, D. L., Zsak, L., 2004. African swine fever virus multigene family 360 genes affect virus replication and generalization of infection in *Ornithodoros porcinus* ticks. J. Virol. 78**,** 2445-53.

Costard, S., Mur, L., Lubroth, J., Sanchez-Vizcaino, J. M., Pfeiffer, D. U., 2012. Epidemiology of African swine fever virus. Virus Res.173, 191-197

de Carvalho Ferreira, H. C., Weesendorp, E., Elbers, A. R., Bouma, A., Quak, S., Stegeman, J. A., Loeffen, W. L., 2012. African swine fever virus excretion patterns in persistently infected animals: A quantitative approach. Vet. Microbiol. 160, 327-340.

Diaz, A. V., Netherton, C. L., Dixon, L. K., Wilson, A. J., 2012. African swine fever virus strain Georgia 2007/1 in Ornithodoros erraticus ticks. Emerg. Infect. Dis. 18**,** 1026-8.

Endris, R. G., Hess, W. R., 1992. Experimental transmission of African swine fever virus by the soft tick *Ornithodoros (Pavlovskyella) marocanus* (Acari: Ixodoidea: Argasidae). J. Med. Entomol. 29**,** 652-6.

Endris, R. G., Hess, W. R., Caiado, J. M., 1992. African swine fever virus infection in the Iberian soft tick, *Ornithodoros (Pavlovskyella) marocanus* (Acari: Argasidae). J. Med. Entomol. 29**,** 874-8.

Estrada-Peña, A., Farkas, R., Jaenson, T. G., Koenen, F., Madder, M., Pascucci, I., Salman, M., Tarres-Call, J., Jongejan, F., 2012. Association of environmental traits with the geographic ranges of ticks (Acari: Ixodidae) of medical and veterinary importance in the western Palearctic. A digital data set. Exp. Appl. Acarol. 59, 351-366.

Farkas, R., Estrada-Peña, A., Jaenson, T. G. T., Pascucci, I., Madder, M., 2013. Basic biology and geographical distribution of tick species involved in the transmission of animal pathogens, including zoonoses. In: Salman, M., & Tarrés-Call, J. (Eds.), Ticks and tick-borne diseases: geographical distribution and control strategies in the Euro-Asia region. CAB International, Wallingford, Oxfordshire, UK, pp 6-26.

Fourie, J. J., Stanneck, D., Luus, H. G., Beugnet, F., Wijnveld, M., Jongejan, F., 2013 Transmission of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks feeding on dogs and on artificial membranes. Vet. Parasitol. DOI:10.1016/j.vetpar.2013.07.026

Gogin, A., Gerasimov, V., Malogolovkin, A., Kolbasov, D., 2013. African swine fever in the North Caucasus region and the Russian Federation in years 2007–2012. Virus Res. 173, 198-203.

Greig, A., 1972. The localization of African swine fever virus in the tick *Ornithodoros moubata porcinus*. Arch. Gesamte Virusforsch. 39**,** 240-7.

Groocock, C. M., Hess, W. R., Gladney, W. J., 1980. Experimental transmission of African swine fever virus by *Ornithodoros coriaceus*, an argasid tick indigenous to the United States. Am. J. Vet. Res. 41**,** 591-4.

Gubbels, J. M., de Vos, A. P., van der Weide, M., Viseras, J., Schouls, L. M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37**,** 1782-9.

Hess, W. R., Endris, R. G., Haslett, T. M., Monahan, M. J., McCoy, J. P., 1987. Potential arthropod vectors of African swine fever virus in North America and the Caribbean basin. Vet. Parasitol. 26**,** 145-55.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129 Suppl**,** S3-14.

Kleiboeker, S. B., Burrage, T. G., Scoles, G. A., Fish, D., Rock, D. L., 1998. African swine fever virus infection in the argasid host, *Ornithodoros porcinus porcinus*. J. Virol. 72**,** 1711-24.

Kleiboeker, S. B., Scoles, G. A., Burrage, T. G., Sur, J., 1999. African swine fever virus replication in the midgut epithelium is required for infection of *Ornithodoros* ticks. J. Virol. 73**,** 8587-98.

Kovalenko, Y. R., Sidorov, M. A., Burba, L. G., 1967. II. Pasture ticks and *Haematopinus* as possible reservoirs and vectors of African swine fever. Trudy Vsesoyuznogo Instituta Eksperimental'noi Veterinarii. 33, 91-94.

Kröber, T., Guerin, P. M., 2007a. An in vitro feeding assay to test acaricides for control of hard ticks. Pest Manag. Sci. 63**,** 17-22.

Kröber, T., Guerin, P. M., 2007b. In vitro feeding assays for hard ticks. Trends Parasitol. 23**,** 445-9.

Labuda, M., Nuttall, P. A., 2004. Tick-borne viruses. Parasitology 129 Suppl**,** S221-45.

Mebus, C. A., Dardiri, A. H., Hamdy, F. M., Ferris, D. H., Hess, W. R., Callis, J. J., 1978. Some characteristics of african swine fever viruses isolated from Brazil and the Dominican Republic. Proc. Annu. Meet. U. S. Anim. Health Assoc. 82, 232-6.

Medlock, J. M., Hansford, K. M., Bormane, A., Derdakova, M., Estrada-Peña, A., George, J. C., Golovljova, I., Jaenson, T. G., Jensen, J. K., Jensen, P. M., Kazimirova, M., Oteo, J. A., Papa, A., Pfister, K., Plantard, O., Randolph, S. E., Rizzoli, A., Santos-Silva, M. M., Sprong, H., Vial, L., Hendrickx, G., Zeller, H., Van Bortel, W., 2013. Driving forces for changes in geographical distribution of *Ixodes ricinus* ticks in Europe. Parasit. Vectors 6**,** 1-11.

Mellor, P. S., Kitching, R. P., Wilkinson, P. J., 1987. Mechanical transmission of capripox virus and African swine fever virus by *Stomoxys calcitrans*. Res. Vet. Sci. 43**,** 109-12.

Mellor, P. S., Wilkinson, P. J., 1985. Experimental Transmission of African Swine Fever Virus by *Ornithodoros-Savignyi (Audouin)*. Res. Vet. Sci.. 39**,** 353-356.

Montgomery, R. E., 1921. On a form of swine fever occurring in British East Africa (Kenya Colony). J. Comp. Pathol. Ther. 34, 159–191.

OIE, WAHID interface animal health information. Office International des Epizooties–World Animal Health Information Database (WAHID) Interface. Available at: http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home. Last Accessed: June 2013.

Plowright, W., 1977. Vector transmission of African swine fever virus. In: Commission of the European Communities Seminar on Hog Cholera/Classical Swine Fever and African Swine Fever. Report EUR 5903 EN, pp 575–587

Plowright, W., Perry, C. T., Peirce, M. A., 1970a. Transovarial infection with African swine fever virus in the argasid tick, *Ornithodoros moubata porcinus*, Walton. Res. Vet. Sci. 11**,** 582-4.

Plowright, W., Perry, C. T., Peirce, M. A., Parker, J., 1970b. Experimental infection of the argasid tick, *Ornithodoros moubata porcinus*, with African swine fever virus. Arch. Gesamte Virusforsch. 31**,** 33-50.

Plowright, W., Thomson, G. R., Neser, J. A.,1994. African swine fever. In: Coetzer, J. A. W., Thomson, G. R., & Tustin, R. C., (Eds.), Infectious Diseases of livestock with special reference to Southern Africa. Oxford University Press, Cape Town, South Africa, pp. 567-599.

R Development Core Team, (2008): A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org

Rennie, L., Wilkinson, P. J., Mellor, P. S., 2000. Effects of infection of the tick *Ornithodoros moubata* with African swine fever virus. Med. Vet. Entomol.. 14**,** 355-360.

Rowlands, R. J., Michaud, V., Heath, L., Hutchings, G., Oura, C., Vosloo, W., Dwarka, R., Onashvili, T., Albina, E., Dixon, L. K., 2008. African swine fever virus isolate, Georgia, 2007. Emerg. Infect. Dis. 14**,** 1870-4.

Sanchez Botija, C., 1963. Reservoirs of ASFV: a study of the ASFV in arthropods by means of haemadsorption. Bull. Off. Int. Epizoot. 60 895–899.

Sonenshine, D. E., 1991. Biology of ticks. Oxford University Press, New York.

Terpstra, C., Wensvoort, G., 1986. [African swine fever in the Netherlands]. Tijdschr. Diergeneeskd. 111**,** 389-92.

Thomson, G. R., 1985. The epidemiology of African swine fever: the role of free-living hosts in Africa. Onderstepoort J. Vet. Res. 52**,** 201-9.

Vial, L., Wieland, B., Jori, F., Etter, E., Dixon, L., Roger, F., 2007. African swine fever virus DNA in soft ticks, Senegal. Emerg. Infect. Dis. 13**,** 1928-31.

Wilkinson, P. J., Lawman, M. J., Johnston, R. S., 1980. African swine fever in Malta, 1978. Vet. Rec.. 106**,** 94-7.

Zeileis, A., Kleiber, C., Jackman, S., 2008. Regression Models for Count Data in R. J. Stat. Softw. 27**,** 1-25.

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., Smith, G. M., 2009. Mixed effects models and extensions in ecology with R. Springer, New York.

Legends of figures

Fig. 1. Boxplots with the minimum, lower quartile, median and upper quartile and maximum observed titres (in $\log_{10}$TCID$_{50}$eq./tick) per week of ticks infected in vitro with the OURT88/1, LIV13/33, and Georgia 2007/1 isolates .Outliers (values greater than 1.5 times the interquartile range) are indicated with a circle, extreme outliers (values greater than 3 times the interquartile range) are indicated with an asterisk. Note: Negative results were considered to be below qPCR detection threshold (-1.5 $\log_{10}$TCID$_{50}$eq./tick)

Fig. 2. Boxplots with the minimum, lower quartile, median and upper quartile and maximum observed titres (in $\log_{10}$TCID$_{50}$eq./tick) per week of ticks infected in vitro with the Malta'78, Netherlands'86, and Brazil'78 isolates. Outliers (values greater than 1.5 times the interquartile range) are indicated with a circle, extreme outliers (values greater than 3 times the interquartile range) are indicated with an asterisk. Note: Negative results were considered to be below qPCR detection threshold (-1.5 $\log_{10}$TCID$_{50}$eq./tick)

Table 1. Viruses used for acquisition feeding.

ASFV isolate	Matrix	ASFV titre ($\log_{10}$TCID$_{50}$)	Reference
OURT 88/1	Spleen homogenate	5.4	(Boinas et al., 2004)
LIV 13/33	Spleen homogenate	5.5	(Rennie et al., 2000)
Georgia 2007/1	Defibrinated blood	5.9	(Rowlands et al., 2008)
Malta'78	Defibrinated blood	5.6	(Wilkinson et al., 1980)
Netherlands'86	Defibrinated blood	5.4	(Terpstra and Wensvoort, 1986)
Brazil'78	Defibrinated blood	5.4	(Mebus et al., 1978)

Table 2. Total number of ticks sampled and number of positive ticks, per week after feeding, for each ASFV isolate/ tick combination.

Tick	Week	OURT88/1		LIV13/33		Georgia 2007/1		Malta'78		Netherlands'86		Brazil'78	
		Total	Pos.	Total	Pos.	Total	Pos.	Total	Pos.	Total	Pos.	Total	Pos.
	0	10	9	10	10	10	10	10	10	10	10	10	10
	1	10	9	10	10	10	9	10	10	10	10	10	10
O.	2	0	0	0	0	0	0	3	3	3	3	0	0
moubata	3	6	6	6	6	6	6	0	0	0	0	6	6
	4	0	0	0	0	0	0	3	3	3	3	0	0
	7	5	4	5	5	5	5	5	3	5	4	5	1
	0	10	10	10	10	10	10	10	9	10	9	10	10
	1	15	15	14	14	11	10	14	14	19	18	16	16
	2	5	4	4	4	2	2	4	4	10	10	6	6
I. ricinus	3	6	4	7	6	3	2	4	4	12	8	7	5
	4	6	5	5	5	3	2	5	5	12	11	7	5
	5	6	4	6	6	2	2	8	8	12	10	7	6
	6	7	7	7	6	3	3	6	5	12	11	7	7
	1	3	2	3	1	3	1	1	0	3	3	4	1
	2	3	3	3	3	3	3	2	2	3	3	3	1
D.	3	13	6	11	5	9	4	4	0	7	5	8	1
reticulatus	4	2	2	4	2	2	1	3	1	5	3	3	0
	5	2	1	2	1	1	1	2	0	1	1	1	0
	6	2	1	1	1	3	2	3	1	2	1	0	0
	8	1	1	0	0	0	0	1	0	1	1	0	0

Total, total number of ticks sampled.

Pos., number of ticks tested positive in qPCR.

Table 3. Resulting rate ratios (RR) [95% CI] of each tick species/ASFV isolate combination on (A) virus uptake during feeding, and (B) overall change of virus concentrations in ticks in subsequent weeks, in comparison with the reference (*O. moubata*, infected with OURT88/1). Significant differences in RR between ticks within each ASFV isolate are shown by different letters. Significant differences in RR between ASFV isolates within each tick species are shown by different numerical digits. For each tick species/ASFV isolate combination, a significant effect of time is shown by ‡.

Period	ASFV isolate	Rate ratio (RR) [95% confidence interval]		
		O. moubata	*I. ricinus*	*D. reticulatus*
A After feeding (week 0)*	OURT88/1	Reference[a,1]	0.77 [0.66; 0.90][b,1]	0.51 [0.36; 0.73][c,1]
	LIV13/33	0.99 [0.85; 1.15][a,1]	0.79 [0.68; 0.93][b,1]	0.58 [0.37; 0.90][c,1]
	Georgia 2007/1	1.05 [0.90; 1.22][a,1]	0.78 [0.66; 0.92][b,1]	0.66 [0.44; 1.00][b,1]
	Malta'78	1.02 [0.87; 1.19][a,1]	0.72 [0.61; 0.84][b,12]	0.40 [0.17; 0.92][b,1]
	Netherlands'86	0.87 [0.75; 1.02][a,1]	0.62 [0.52; 0.72][b,2]	0.14 [0.08; 0.22][c,2]
	Brazil'78	0.89 [0.75; 1.04][a,1]	0.83 [0.72; 0.97][a,1]	0.71 [0.31; 1.63][a,1]
B For each additional week**	OURT88/1	0.98 [0.94; 1.02][a,2]	0.96 [0.93; 1.00][a,1]‡	0.99 [0.90; 1.08][a,12]
	LIV13/33	1.04 [1.01; 1.08][a,1]‡	0.96 [0.92; 0.99][b,1]‡	1.00 [0.88; 1.13][ab,12]
	Georgia 2007/1	0.89 [0.86; 0.93][a,3]‡	0.93 [0.88; 0.98][a,1]‡	0.96 [0.86; 1.07][a,2]
	Malta'78	0.93 [0.88; 0.97][a,23]‡	0.94 [0.90; 0.98][a,1]‡	0.99 [0.79; 1.23][a,12]
	Netherlands'86	0.98 [0.94; 1.02][b,2]	0.94 [0.91; 0.98][b,1]‡	1.17 [1.05; 1.31][a,1]‡
	Brazil'78	0.96 [0.90; 1.02][a,23]	0.95 [0.91; 0.98][a,1]‡	0.96 [0.66; 1.41][a,12]

* corresponds to the uptake of virus, relative to the reference; higher RR signify more virus uptake, lower RR less virus uptake.

** corresponds to the effect of time (in weeks, continuous variable); a RR>1 corresponds to an overall increase of virus in time, an IR<1 to an overall decrease of virus in time.