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African swine fever virus infection in *Ornithodoros* ticks corrected copy

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ABSTRACT

African swine fever virus (ASFV) is an arbovirus which is vectored by soft ticks of the *Ornithodoros* spp. and in the sylvatic cycle infects wart hogs and bush pigs. ASFV infection of domestic swine causes a high mortality disease. On the other hand, ASFV infection of the tick can result in a high-titered and persistent infection depending upon the ASFV isolate and the tick combination. Recently, morphological, classical virology (titration) and recombinant ASFV have been used to study the cellular, molecular and genetic interactions that occur between ASFV and its host tick.

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1. Introduction

African swine fever virus (ASFV) infects its vector tick *Ornithodoros* spp. and produces a high-titered and persistent infection. ASFVs very large genome, approximately 180 kb, encodes approximately 160 proteins. Many of the proteins' function are unknown and probably influence ASFV survival and growth in the tick. ASFV is the only known double stranded DNA arbovirus and is the single member of the Asfarviridae (Dixon et al., 2005). ASFV infects warthogs (*Phacochoerus aethiopicus*), giant forest hogs (*Lylochoerus meinertzhageni*) and bush pigs (*Potamochoerus* spp.) and causes minimal disease (DeTray, 1957; Anderson et al., 1998) but causes high mortality in domestic swine (*Sus domesticus*) (Plowright et al., 1994; Costard et al., 2012). In sub-Saharan Africa, the argasid tick, *Ornithodoros porcinus porcinus* (Walton) serves as the ASFV vector and host and, in this sylvactic cycle, ASFV passes between ticks living in warthog burrows and juvenile warthogs (Plowright et al., 1969a,b). The sylvatic cycle can continue in the absence of transmission or acquisition feeding with trans-stadial, venereal and transovarial transmission of the virus in the tick population (Plowright et al., 1970a,b, 1974).

ASFV was first isolated in Spain in the 1960s in *O. erraticus* (Sanchez-Botija, 1963). Early studies of ASFV experimental infection of ticks showed primary localization to the midgut (Greig, 1972) and hemocytes (Endris et al., 1987) with relative heterogeneity amongst different geographic isolates (Plowright et al., 1970a,b; Kleiboeker and Scoles, 2001). ASFV-tick-swine infection studies demonstrated transmission with African (*O. savignyi*), North American (*O. coriaceus*, *O. turicata*) and Caribbean ticks (*O. puertoicensis*) (Groocock et al., 1980; Mellor and Wilkinson, 1985; Hess et al., 1987, 1989).

The cell biology of ASFV infection and the genetics of ASFV virulence and host range determinants have been dissected elegantly using microscopy, biochemistry and reverse genetics in the last four decades (Hawes et al., 2008; Tulman et al., 2009; Suarez et al., 2010a,b). Light and electron microscopy, classical virology and ASFV recombinants have been used to follow the structural and viral consequences of tick infection (Kleiboeker and Scoles, 2001).

2. *Ornithodoros* anatomy and physiology

As an arbovirus, ASFVs infection, replication, generalization, persistence, and transmission are adapted to the tick's anatomy and physiology (Weaver, 2006; Labuda and Nuthall, 2004; Nuthall, 2009; Kaufman, 2010). The cuticle of *Ornithodoros*, for example, expands 3- to 5-fold during the very rapid uptake of the blood meal (Sonenshine, 1991) (Fig. 1). Although the organs that are usually infected (midgut, coxal and salivary glands) are in very close proximity to one another (Fig. 2), there are significant cellular barriers to viral spread (Kleiboeker et al., 1998, 1999; Nuthall, 2009). During feeding, the tick midgut receives a large volume of blood filling its' many diverticula (Grandjean and Aeschlemann, 1973; Askov, 1982; Grandjean, 1983, 1984; Sonenshine, 1991) (Figs. 2 and 3). The first phase of *Ornithodoros* blood meal intercellular digestion is the uptake of erythrocytes by the midgut digestive cells (Figs. 3A,B, and 4). One week later there is a rapid proliferation of undifferentiated midgut cells to allow for the adsorption and digestion of the remaining blood meal (Fig. 3C). Three weeks post feeding undifferentiated midgut cells continue to increase in size and number and the hematin (an accumulation of iron remaining after protein portion of hemoglobin molecule is digested) filled digestive cells are sloughed into the lumen (Fig. 3D). Only a few

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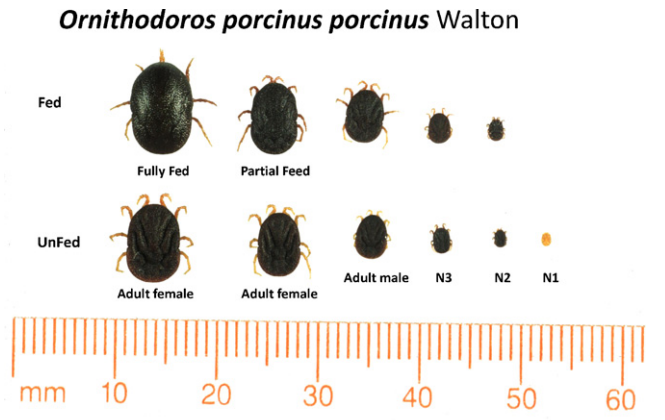

Fig. 1. Fed and unfed nymphal and Adult *Ornithodoros porcinus porcinus* ticks from colonies maintained on Plum Island Animal Disease Center 1995–2004. Adults and nymphs were collected from warthog burrows in Kruger National Park South, Africa and on farms in the Transvaal, South Africa. The ticks in the top row were fed through a membrane on heparinized swine blood for 45 min at 41 °C and all were fixed in 70% ethanol. The engorged female is approximately 3 times the size of the unfed female.

intact digestive cells remain after 6 months and the single muscle layer appears to squeeze the diverticula (Fig. 3E). Uptake of individual erythrocytes occurs by extension of the digestive cell microvilli with accumulation within large phagosomes (Fig. 4). The mechanism(s) for intracellular digestion is(are) not well known (Sonenshine, 1991). Cathepsin D, acid phosphatase, and phospholipase, however, have been identified in the digestive cell lysosome (Askov, 1982; Grandjean, 1984; Sonenshine, 1991).

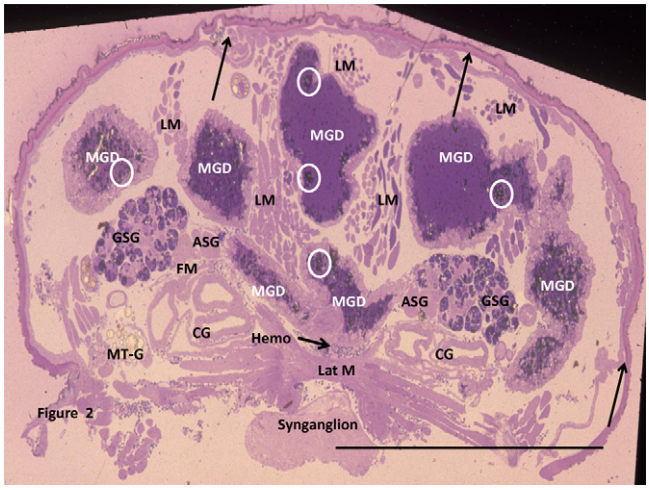

Fig. 2. A light micrograph of nymphal stage 2 tick 3 weeks after a blood meal. The tick has finished generating a new cuticle and is beginning to molt (arrows). The 1 μm-thick resin cross section from the tick's anterior portion was stained with 2% Toluidene Blue. The thin-walled midgut diverticula (MGD) contain phagocytic cells with dark areas which are the remains of intercelluarly digested eyrthrocytes or hematin (circles). The individual hematin crystals are too small to be seen at this magnification, see Figs. 3 and 4). Below the MGD are the paired salivary glands composed of the agranular (ASG) and the granular (GSG) portions. Beneath the SG are the tubules of the paired coxal glands (CG) along with its' kidney-like filtration membrane (FM). Trapped between the lowest MGD and the lateral muscle are hemocytes (Hemo with arrow). White crystals, presumably guanine, are present in the nitrogenous excretory system, the Malpigihian tubules (MT-G). The tick brain or synganglion occurs under the laterally disposed muscles (Lat M). Other muscle bundles traverse longitudinally (LM) and are attached to the exoskeleton. Bar is 0.5 mm.

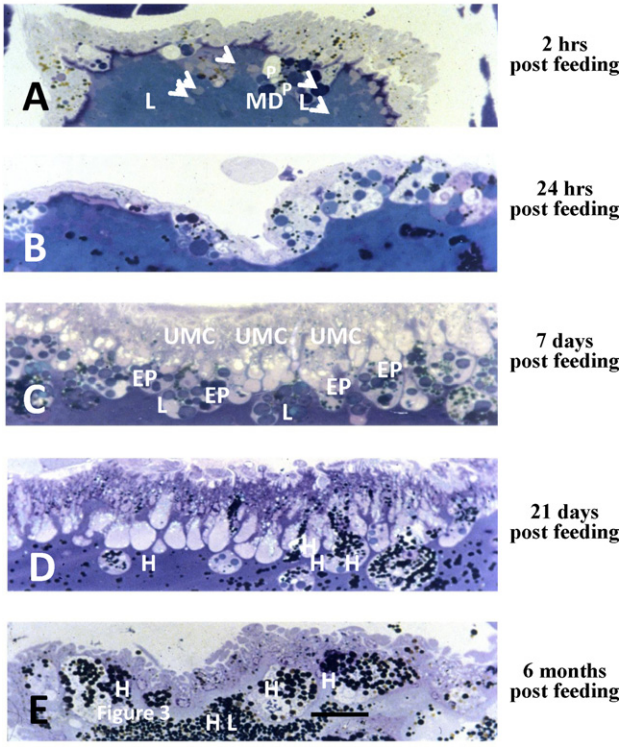

Fig. 3. Light microscopy of the uptake and intercellular digestion of a meal blood in nymphal ticks. Stage two nymphs were allowed to feed to repletion on swine blood containing with 1×10^6 HAD_{50} of ASFV Pret 4 and then processed for electron microscopy after 2 h, 24 h, 7 days, 21 days, and 6 months. The sections are 1 μm thick and are stained with 2% Toluidine Blue. (A) Swine erythrocytes (arrows) in the tick midgut lumen (L) after 2 h. A few of the differentiated digestive midgut cells (DM) which remain after the digestion of the first blood meal have erythrocytes in phagolysomes (P). (B) After 24 h, erythrocyte uptake continues among the differentiated cells. (C) After 1 week the undifferentiated midgut cells (UMC) have proliferated and are pushing the digestive cells which have their phagolysosomes filled with erythrocytes (EP) into the lumen (L). (D) By 3 weeks, the undifferentiated midgut cells (UMC) are the major component of the midgut wall and the digestive cells with their many hematin crystals (the iron core remains of the digested hemoglobin) are sloughed into the lumen (L). (E) At 6 months post feeding only a few hematin-containing cells remain intact while the majority have lysed and their hematin core are in the lumen (HL). The bar is 2.5 μm.

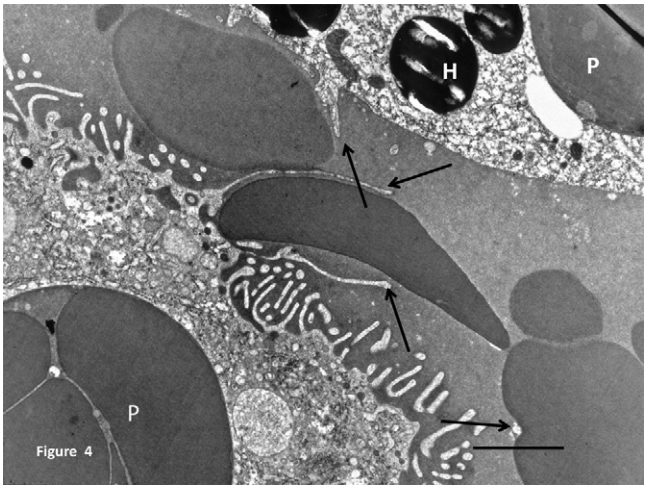

Fig. 4. Electron micrograph of the uptake of red blood cells by microvilli of the digestive cells (arrows). Phagolysosomes containing multiple red blood cells (P). Hematin crystal (H). Bar is 2 μm.

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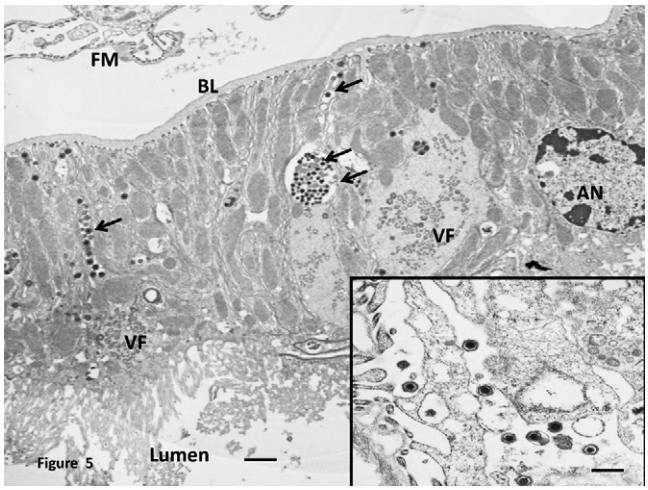

Fig. 5. Electron micrograph of coxal gland after ASFV infection. Mature ASFV array at the margins of the cell (arrows). A thick basal lamina (BL) covers the mitochondria-rich tubule portion and microvilli project into the lumen of the gland. Extensive virus factories (VF) and minor vesicle formation occur in the cytoplasm. A nucleus with condensed and marginated chromatin occurs with the ASFV infected cell (AN). The bar is 1 μm. Filtration membrane (FM). Inset. A high magnification view of budded ASFV behind the podocytes in the filtration membrane. The bar is 500 nm.

In ASFV vector ticks, the blood meal is concentrated by elimination of water using paired coxal glands [named for the gland's discharge opening between the first segment (coxal) of the first and second pair of legs] located beneath the salivary glands (Fig. 2) (Sonenshine, 1991). Very large volumes of coxal fluid are expressed during feeding (Sonenshine, 1991). A coiled tubular system, richly supplied with mitochondria, retains hemolymph and other molecules (Fig. 5) (Sonenshine, 1991). The convoluted filtration membrane provides a large surface to regulate water and ions (Sonenshine, 1991) (Fig. 5, inset).

Tick salivary glands facilitate the feeding process through a complement of enzymes, hemostatic and inflammatory compounds, and modulators of blood flow (Sonenshine, 1991; Mans, 2002; Mans et al., 2008). The *Ornithodoros* salivary glands are very complex, large and paired structures typified by the presence of both granular and agranular and cell types (Sonenshine, 1991; Mans, 2002). The agranular cell type is thought to be involved in water regulation between feedings (Sonenshine, 1991). The heterogeneous granules in the granule cell type store the very large number of pharmacologically active compounds found in the saliva (Fig. 6A and B). Morphologically, there are four histochemically distinct staining granules (Mans et al., 2004). Many of the constitutive proteins of *Ornithodoros* granules and those involved in granule biogenesis have been described, for example, a platelet aggregation inhibitor, savignygrin, lipocalin, and apyrase (Mans, 2002; Mans et al., 2001; Mans and Neitz, 2004; Mans and Ribeiro, 2008a,b). In addition, an analysis of the sialome transcriptosome of soft ticks reveals genes of the lipocalin family, serine protease inhibitors, insulin growth factor-binding protein, and adrenomedulin (Francischetti et al., 2008). The peptidase-inhibitory, immunomodulatory protein, systatin, has been resolved at 2.45 Å and its function characterized (Salat et al., 2010).

Argasid ticks such as *Ornithodorus* spp. reproduce sexually beginning shortly after feeding (Sonenshine, 1991). Oocytes, for example, are produced in an ovary supported by funicular cells (Fig. 7), and are conducted through oviducts, connecting tubules into a muscular cervical vagina and eventually the eggs are coated with waxy secretions from Gene's organ during oviposition (Sonenshine, 1991). Tick spermatids (Fig. 8B) are found in paired testes surrounded by accessory glands (Fig. 8A) whose probable function is to provide secretions for the spermatophore and for the capacitation of spermatoza (Sonenshine, 1991).

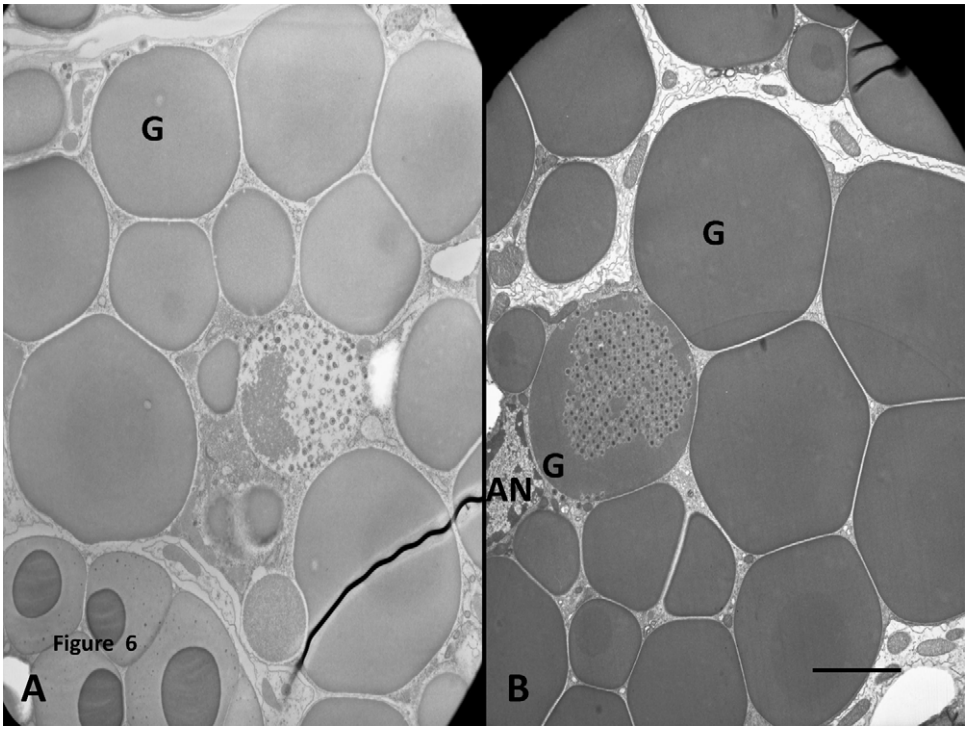

Fig. 6. Incorporation of ASFV into granules of the salivary gland or "molecular crowding". (A) Virions from the adjacent factory appear to be incorporated into a granule along with the salivary gland matrix protein. (B) A mature granule (G) with a crystalline array of ASF virions. The salivary gland cell nucleus shows marginated chromatin (AN) and some evidence of cytoplasmic disruption. The bar is 2 μm.

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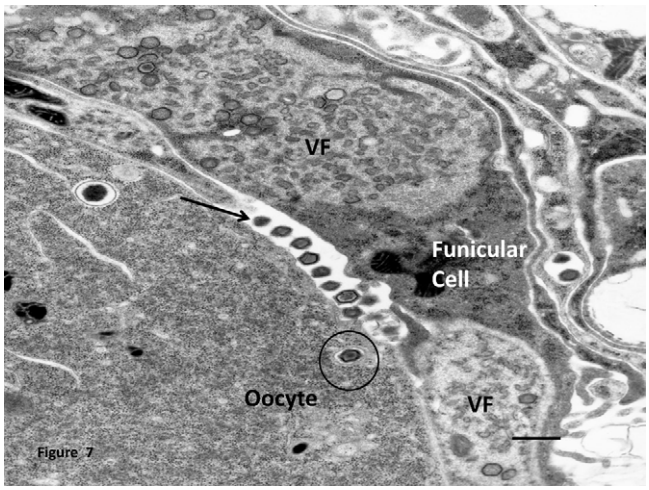

Fig. 7. ASFV in female tick reproductive organ. The oocyte from an infected tick is adjacent to a funicular cell with virus factory (VF). Virions without plasma membranes occur between the cells (arrow). There were more mature virions in both the oocyte and the support cell in other sections. The encircled virion may be being taken up by the oocyte or may be at the surface resting in a depression. The bar is 500 nm.

3. ASFV pathogenesis in ticks

3.1. Infection

Successful ASFV infection of the tick and its eventual transmission during a subsequent blood meal depends upon the discrete molecular interactions between the virus and the tick (Nuthall, 2009). Infection of the midgut cells is the first barrier (Kleiboeker et al., 1998; Nuthall, 2009). In the viremic domestic swine, greater than ninety percent of ASFV infectivity associates with the red blood cell (RBC) fraction (Wardley and Wilkinson, 1977; Borca et al., 1998) and the virus particle firmly associates with the plasma membrane usually in a small depression (Quintero et al., 1986). Kleiboeker et al. (1998) show a degrading ASFV particle attached to a RBC in digestive cell phagosomes 72 h post-feeding. Twenty one days post feeding, many of the digestive cells are positive by anti-ASFV monospecific antibody immunohistochemistry (Kleiboeker et al., 1999) or have extensive viral factories (Burrage et al., 2004) suggesting that initial midgut infection comes from uptake of RBC with adsorbed ASFV. Recently, Rowlands et al. (2009) have shown that the ASFV CD2v protein associated with RBC attachment (Rodríguez et al., 1993; Borca et al., 1998) increases the replication in the tick midgut. The requirement of intact or disrupted RBCs for tick midgut infection, however, was also tested using ASFV negative colonized ticks obtained from a South African site (Pretoriuskop 4) ASFV negative offspring were membrane-fed either heparinized swine blood or fetal bovine serum spiked with an ASFV isolate from the same geographic site. Twenty days post feeding, the ASFV titers in ticks fed by either inoculum were not significantly different (Kleiboeker et al., 1999).

The receptor-mediated uptake of ASFV into mammalian tissue culture cells (Vero) has been well demonstrated (Alcamí et al., 1989, 1992). Hernaez and Alonso (2010) also demonstrate that in culture cells clathrin and dynamin were required for ASFV uptake complimenting a receptor-mediated mechanism. On the other hand, the macropinocytosis pathway was shown also to be effective in uptake of a tissue culture adapted ASFV (Sanchez et al., 2012). Primary swine macrophages do not rely on a receptor-mediated uptake but do require microtubules (Basta et al., 2010). Unlike tissue culture cells, cell surface receptors for ASFV may or may not be required as bulk phagocytosis and fluid-phase pinocytosis are part of tick midgut digestion pathway (Sonenshine, 1991).

3.2. Factory formation, intercellular and intracellular transport

In tissue culture, ASFV-infected cells show a role for the filamentous protein vimentin in the initiation and formation of a protected space for ASFV particle assembly (Carvalho et al., 1988;

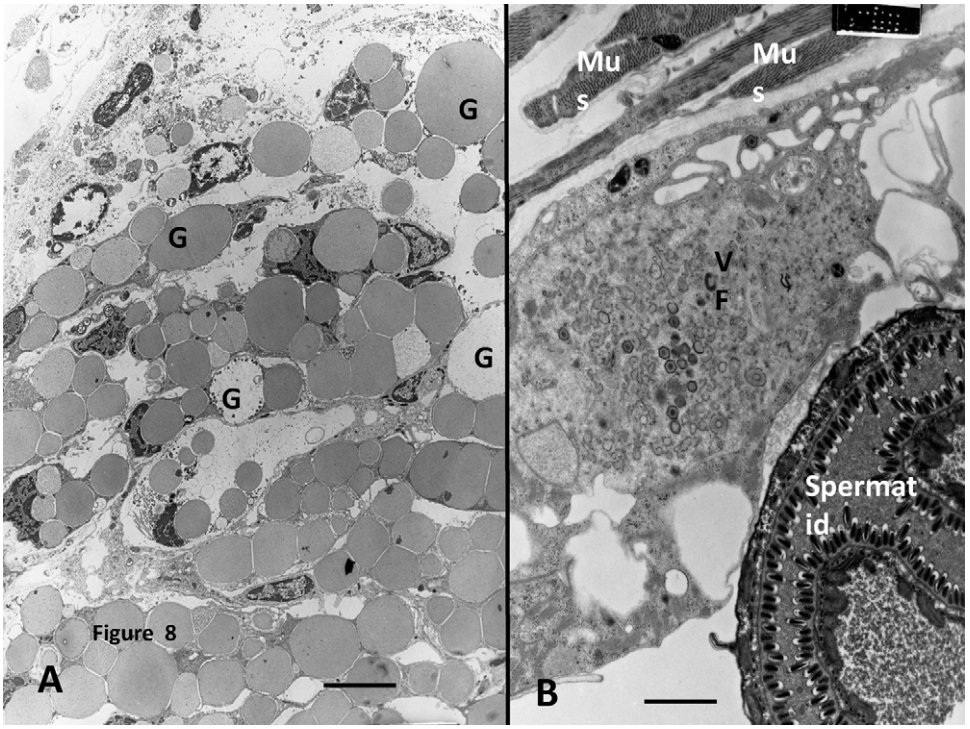

Fig. 8. ASFV in male tick reproductive organs. The secretory vesicle-filled accessory gland has ASF virus within granule (G) or in the cytoplasm. One of the cubodial cells lining the vasa diferentia has a prominent virus factory (VF). A mature spermatid is closely associated with this cell. Muscle (Mus). The bar for (A) is 5 μm and for (B) is 1 μm.

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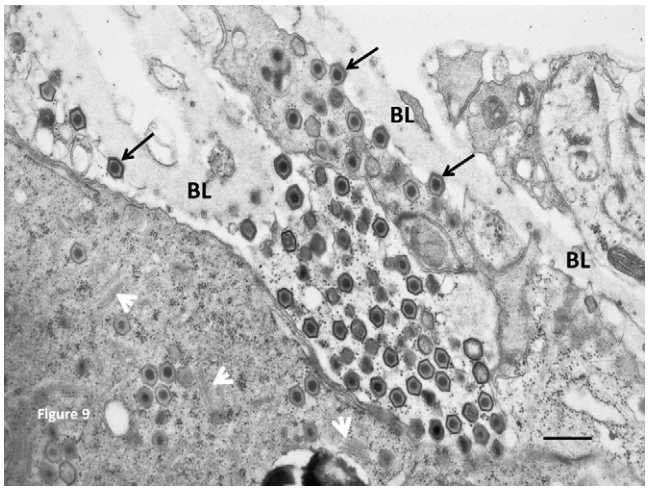

Fig. 9. A tangential section through ASFV pooling underneath the basal lamina of the undifferentiated midgut cells at 21 days post-feeding. ASFV is budding into the lamina, deforming it (black arrows). Mature virions are near the surface and bundles of intermediate size filaments occur (white arrowheads). Bar is 500 nm.

Stefanovic et al., 2005). In ticks, the first indication of ASFV replication is the appearance at 72 h post feeding of membrane crescents in a homogeneous, less electron dense region of digestive cells that are also devoid of ribosomes, vesicles, microtubules and filaments (Kleiboeker et al., 1998). Empty and full ASFV particles occur in similar homogeneous regions at later times in both midgut cells (Fig. 9) and hemocytes (Fig. 10). The presence or absence of vimentin has yet to be definitively determined for arthropods. However, bundles of short uniform filaments are often seen at the edges of the virus factory (Fig. 9), and they are ultrastructurally similar to intermediate filaments. These filaments are, however, not identical to in terms of length and bundle organization to those occurring in ASFV infected swine macrophages (Burrage, unpublished data).

Intercellular transport of both vimentin and mature ASFV particles is dependent in tissue culture cells upon cellular microtubules as shown by colchicine, taxol and nacodozole studies (de Matos and Carvalho, 1993; Alonso et al., 2001) and by immunofluorescence (Jouvenet et al., 2004; Stefanovic et al., 2005). Conventional

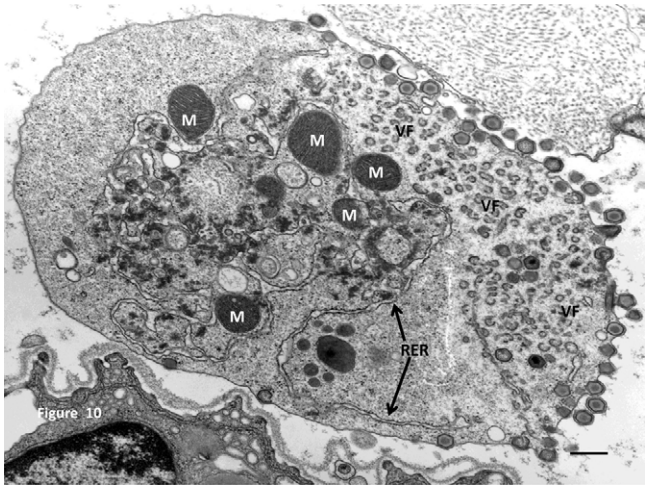

Fig. 10. Replication of ASFV in a hemocyte. An ASFV infected type 1 hemocyte has the uniform, organelle-free cytoplasmic region of a virus factory (VF). The factory is filled with membrane crescents, empty hexagons, and developing and mature particles. Rough endoplasmic reticulum (RER) has proliferated and mitochondria (M) are closely associated with the factory. Mature virions with centered cores emerge from the hemocyte periphery acquiring a plasma membrane coat. A nearby connective tissue cell has a basement membrane. The bar is 500 nm.

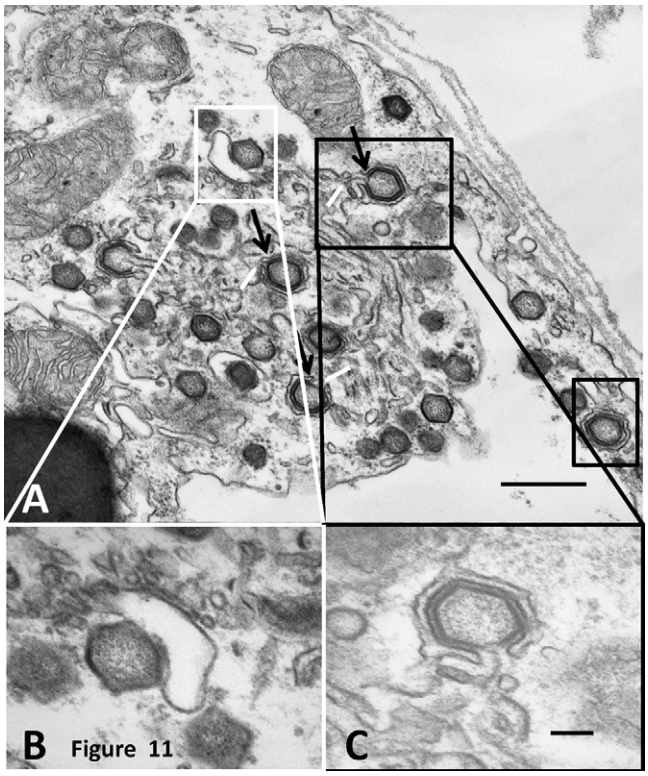

Fig. 11. Viral morphogenesis in a desiccated tick. At 9 months post feeding, a connective tissue cell surrounding the salivary gland has a clear virus factory with membrane crescents, a ring of mitochondria (M) showing no signs of degeneration, and full and empty hexagonal profiles. At one vertex of some of hexagons black (arrows), there is an opening filled with a bundle of uniformly thin threads (white solid arrows) (A and C). Small cytoplasmic vesicles appear to be fusing into a larger vesicle which in turn appears to be associating with the filling hexagon (A in the box and enlarged in B). As seen in (B), the large membrane vesicle can be just associating with the hexagon, and in (C) encirculating and shrinking to enclose the hexagon except in the opening. The bar for (A) is 500 nm and for (B) and (C) is 100 nm.

kinesin acts with microtubules to carry the virus particles to the plasma membrane (Jouvenet et al., 2004). Although tick pathogenesis and classical tick fine structure studies were not optimized for the preservation and/or presentation of microtubules, only a few microtubules were seen (Balashov, 1983; Sonenshine, 1991; Kleiboeker et al., 1998, 1999; Burrage et al., 2004). Given the very large size (>200 nm) of the ASFV particle and the high density of the cultured cell cytoplasm, molecular motors may be necessary to translocate the ASFV particles to the plasma membrane (Alonso et al., 2001; Jouvenet et al., 2004). Unlike the central location of the virus factory in the cultured cells, the tick's virus factory and maturing virions are found subjacent to the plasma membrane (Figs. 9 and 11) (Kleiboeker et al., 1998, 1999). Since tick ASFV transport appears to cover short intracellular distances, an extensive microtubule/kinesin network may not be necessary as shown for ASFV replication in tissue culture (Jouvenet et al., 2004). However, tick ASFV virus exocytosis may require actin filaments (Jouvenet et al., 2006) and a manner similar to the closely related vaccina virus (Cudmore et al., 1995). In addition to the vimentin cage, ASFV infection in tissue culture causes a re-location of mitochondria to surround the aggresome-assembling virus (Rojo et al., 1998; Heath et al., 2001). In uninfected tick midgut cells, the mitochondria are randomly distributed in the cytoplasm whereas in infected cells mitochondria ring the factory (Fig. 10). In cultured cells, the trans-Golgi network (TGN) is lost post-ASFV infection (McCrossan et al., 2001); however, the effect on TGN membranes in infected tick cells is unknown.

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There is indirect evidence of lymphoid cell-to-cell ASFV transport in both highly susceptible domestic swine and the relatively resistant bush pig suggested by the uniform localization of ASFV antigen in the germinal center (Oura et al., 1998; Fernandez de Marco et al., 2007). In ticks, the occurrence of intracellular transport may be inferred due to the initial infection of the digestive cells followed by the appearance of virus factories in the undifferentiated midgut cells and the occurrence of budding particles at the periphery of the digestive cells. However, it cannot be definitely concluded that the infection of the undifferentiated cells could occur by uptake of particles shed into the lumen or from the residual inoculum.

3.3. Midgut escape barrier

Molecular and mechanical midgut escape mechanisms after amplification have been recently shown for insect viruses (Weaver, 2006; Nacer et al., 2008; Means and Passarelli, 2010; Passarelli, 2011; Clements, 2012), whereas escape mechanism(s) for tick viruses is(are) unknown (Labuda and Nuthall, 2004; Nuthall, 2009). Tick viruses such as ASFV and Dugbe, however, readily infect hemocytes in the surrounding hemolymph (Booth et al., 1991; Kleiboeker et al., 1998, 1999). The soft tick's midgut basal lamina has not been biochemically or cytochemically characterized, unlike some hard ticks and insects (Spielman, 1990; Ge et al., 1996; Passarelli, 2011). By electron microscopy, *Ornithodoros's* basal lamina is a homogeneous and continuous layer covering over the midgut epithelial cells and runs under the muscle network (Sonenshine, 1991; Kleiboeker et al., 1998, 1999). The continuous dense lamina is different than the slightly perforated lamina of the insect Passarelli, 2011. After midgut replication, ASFV particles apparently pool under the lamina (Fig. 9). The apparent entrance of particles into the lamina could be due to the action of matrix metalloproteinases as reported for baculoviruses and insects (Means and Passarelli, 2010). Alternatively, it may be possible that ASFV escape is mechanical with the thin muscle network constricting the midgut diverticula as the blood meal shrinks, causing the budding membrane associated viral particles to be squeezed into the hemocoel where they are picked up by the hemocytes.

3.4. Generalization and the salivary gland barrier

After oral infection, escape from the midgut and hemocyte infection, ASFV must cross into the salivary gland for virus transmission using this route. How arboviruses reach the salivary gland is largely unknown (Nuthall, 2009) and unlike Thogoto virus, there was no structural evidence of synganglion infection by ASFV so that a neural route of virus passage to the gland is unlikely (Booth et al., 1989). Electron microscopy of infected and uninfected ticks reveals that the salivary gland is covered by a thin basal lamina probably secreted by connective tissue cells that surround the gland (Sonenshine, 1991; Kleiboeker et al., 1998). However, ASFV replication occurs in connective tissue cells surrounding other internal organs and in the salivary gland (Kleiboeker et al., 1998). How the connective tissue cells become infected is unknown.

3.5. Transmission

Salivary gland secretions contain approximately 2–3 $\log_{10}$ of ASFV at 56 days post feeding and ASFV virus was detected scattered in granules (Kleiboeker et al., 1998). Once in the cytoplasm of the granule forming cells, condensed ASFV particles and granule protein (Fig. 6A) form an array in the particle center (Fig. 6B). The ASFV crystal array seen in many granules may result from the "molecular crowding" that occurs during granule formation (Mans et al., 2004). A small survey of mature granules, however, reveals that ASFV particles only associated with granules containing uniform, less electron-dense contents (Fig. 6A and B). However, Mans et al. (2004) indicate that different morphologies do not necessarily mean different or singular contents and therefore, the appearance of particles in these granules may be random and not a tropism for a particular protein. Since the granules are expelled during feeding (Coons and Roshdy, 1981), ASFV are delivered into the mammalian blood stream.

During feeding coxal fluid is also expressed and it contains a high concentration of virus (Kleiboeker et al., 1998, 1999). Thirty days post feeding, the filtration membrane has abundant budding and free virions (Fig. 5, inset) and the tubular portion contain cells with extensive viral factories and large numbers of mature virions (Fig. 5). The mechanism by which ASFV gets around or through the basal lamina covering the tubule and/or the podocytes of the filtration membrane to exit through the coxal orifice is currently unknown.

Transovarial ASFV transmission varies significantly and appears to be based on whether the isolate and tick were combined experimentally (Rennie et al., 2001) or were naturally infected (Plowright et al., 1970b). Using a South African isolate and a colony tick from the same region, virus occurs on the surface of the oocyte (Fig. 7).

Sexual transmission occurs between infected males and uninfected females (Plowright et al., 1974). ASFV infects many cells of the accessory gland (Fig. 8A) and occurs in the cells lining the sperm duct (Fig. 8B).

3.6. Virus morphogenesis

The contributions and origins of individual and membranous ASFV structural and non-structural proteins to virus assembly in tissue culture have been the subject of intense microscopic, biochemical and recombinant virus analysis over the past five decades beginning with Breeze and DeBoer (1966) (Andres et al., 1997, 1998; Brookes et al., 1996, 1998a,b; Garcia-Escudero et al., 1998; Hawes et al., 2008; Heath et al., 2001, 2003; Rouiller et al., 1998; Suarez et al., 2010a,b; Wileman, 2007). Unfortunately, the assembly of ASFV in the tick is not as well determined due to the primary focus on the occurrence and distribution of the virus and the difficulty of finding early stages of replication. In well-formed assembly sites, however, as seen in Figs. 5, 9 and 10, virus particles in various stages of maturation occur in a uniform cytoplasmic region bordered by bundles of filaments. The origin of the hexagonal profiles could well be the endoplasmic reticulum as seen for ASFV tissue culture cells (Andres et al., 1998; Rouiller et al., 1998). One clear departure from tissue culture, however, occurs at 9 months post-feeding when the tick is quiescent and desiccated. In Fig. 11, virions appear in the latter stages of assembly with an open vertex and thin filaments entering (Rouiller et al., 1998), however, there are small fusing membrane vesicles intimately associated with a portion of a maturing ASFV particle. In the same region, membranes associate closely with the entire maturing particle (Fig. 11C). The source of these membranes is not known but it is possible that these membranes may provide protection during the potential long periods between feeding for the long-lived tick.

4. Specific ASFV genes and tick replication

Single and multi-gene deletion recombinant ASFV has been used to document host range and virulence determinants in swine (Tulman and Rock, 2001; Tulman et al., 2009). Elimination of one of the two multigene family 360 (genes 3HL, 3IL and 3LL) (Pret4 Δ3-C) results in a 100- to 1000-fold reduction in tick titer after oral infection (Burrage et al., 2004). At 21 days post feeding, examination of the Pret4 Δ3-C infected midgut reveals many fewer intact

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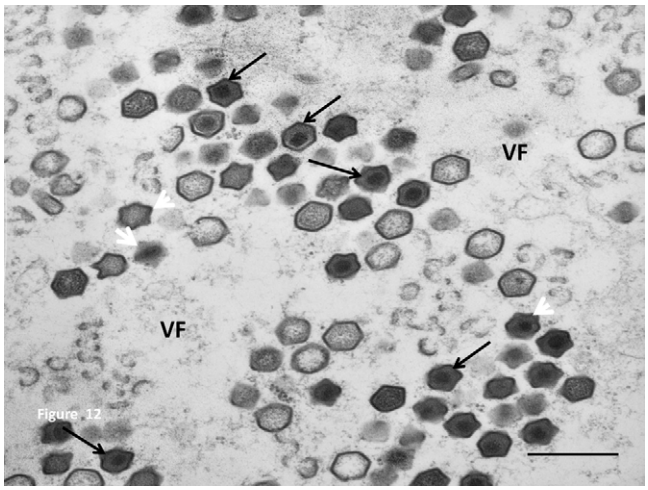

Fig. 12. A midgut cell from a tick infected with the ASFV recombinant virus Pret4-Δ9GL. All the full virions have acentric nucleoids (black arrows) and many have collapsing hexagons (white arrowheads). The bar is 500 nm.

particles, aberrant viral forms, cytoplasmic and nuclear pathology. Deletion of a single gene, 9GL, a yeast ERV1 homolog, dramatically reduces swine virulence and appears in swine macrophage culture to alter virus maturation (Lewis et al., 2000). Infection of ticks with Δ9GL results in an ASFV particle with a similar acentric nucleoid but also a marked deformation of the hexagonal profile (Fig. 12). Studies of the effect of Δ9GL on virus titer are incomplete. Addition of CD2v to non-hemadsorping ASFV isolates increases the tick titer in the presence of RBCs (Rowlands et al., 2009).

5. Influence of ASFV infection on tick survival

The outcome of a tick's exposure to ASFV is remarkably variable. Colony ticks experimentally infected with a tick isolate from the same region results in a high-titered, persistent infection with no gross differences from uninfected ticks with one exception. Infected females will die after laying a second egg mass (Kleiboeker et al., 1998). On the other hand, exposure of European and North American ticks to a variety of ASFV isolates results in high mortality but also occasionally very long term persistence (Endris et al., 1991, 1992a,b; Endris and Hess, 1992; Groocock et al., 1980; Hess et al., 1987). The mechanism of tick death, other than the posited metabolic pressure placed upon adult females laying eggs while carrying a large viral load (Kleiboeker et al., 1998) and the rupture of the gut while taking in an ASFV infected blood meal (Rennie et al., 2000) is unknown. The lack of ASFV generalization in ticks after oral infection with non-adapted isolates, *i.e.* ASFV Malawi grown in South African ticks may be attributed to the premature death of infected digestive cells (Kleiboeker et al., 1999). The reason for long-term clearance from ticks has not been determined (Hess et al., 1989).

Apoptosis, or programmed cell death occurs in swine cells infected with ASFV *in vivo* and *in vitro* (Zsak and Neilan, 2002; Tulman et al., 2009). However in the tick, all cells with a virus factory have a nucleus with marginated and condensed chromatin (Figs. 5 and 6) (Kleiboeker et al., 1998). Interestingly, these cells do not display any other morphological features of apoptosis such as cell shrinkage, cell blebbing, nuclear degranulation and formation of apoptotic bodies as occur with virus-infected insect midgut cells (Zhang et al., 2002; Vaidyanathan and Scott, 2006; Passarelli, 2011). Pioneering work with rationally designed recombinant viruses clearly shows some ASFV proteins inhibit apoptosis in cultured cells (Zsak and Neilan, 2002). 5HL, for example, resembles the proto-oncogene BcL-2, while 4CL appears related to inhibitor of apoptosis genes. The multigene families 360 and 530, however, have no homologies with genes in current databases but deletion of six MGF360 and four MGF 530 results in no ASFV growth in swine macrophages (Zsak et al., 2001). Infection of ticks with deletion of three MGF 360 genes (3HL, 3IL and 3LL) significantly reduced ASFV growth and generalization and infected digestive cells showed nuclear and cytoplasmic changes consistent with apoptotic death (Burrage et al., 2004).

6. Closing statement

ASFVs infection of its vector tick *Ornithodoros* spp. suggests a co-evolution (Nuthall, 2009; Kleiboeker et al., 1998). Further work with cellular and biochemical tools and recombinant viruses will be needed to confirm ASFV adaptation to the tick's physiology, maximal transmission of ASFV during feeding, minimal detrimental effect of ASFV on the host by arresting cell death and ASFV persistence during the possible years between feedings in an arid environment. Pathogenesis studies, however, strongly suggest that ASFV follows the well-described virus-tick pathways: ingestion of blood meal, replication of the virus in the midgut, escape from the midgut, entering the hemocoel and infecting the major secretory gland, the coxal and salivary glands (Nuthall, 2009). The tick and virus appear be very well suited for ASFV replication using, for example, the tight association of the virus with the red blood cell to gain entry into the midgut cell. The virus replicates very successfully in the midgut cells, releasing virus for infection of the other cells and more importantly, is taken up by the undifferentiated cells and masses many virions at the basal lamina. The escape from the midgut is still not clear but the efficient replication in the circulating hemocytes indicates a means of transport to the coxal and salivary glands. The minimal disruptive nature of the replication in the coxal and salivary glands allows for the maximum transmission as these fluids are released during feeding on the host. The crystalline inclusion of virions in the salivary gland granules delivers a very high concentration of mature virions into the blood stream where the reticulo-endothelium system could pick them up and provide for efficient replication in the host. The co-evolution of ASFV and *Ornithodoros* has apparently led to the virus's adaptation to the tick's longevity and it's arid environment.

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