Mosquito-borne arboviruses initiate infection in the midgut of the mosquito following ingestion of a viraemic blood meal. As the infection progresses, the virus replicates and disseminates from the midgut then invades and replicates in the salivary glands. There is little information regarding dissemination of virus from the midgut to the salivary glands. It is postulated that the virus requires (i) a means to amplify after its escape from midgut epithelial cells, so that it can infect the salivary glands efficiently (Girard et al., 2003, 2004; Infanger et al., 2004), and (ii) a vehicle to ensure dissemination from the midgut to the salivary glands (Romoser et al., 2004).

In lepidopteran larvae infected with baculoviruses, virus entry into midgut epithelial cells is followed by infection of the tracheoles, the oxygen transporting tissue in insects. In several lepidopteran species, insect blood cells, called haemocytes, facilitate amplification of baculoviruses as well as virus dissemination from tracheoles to secondary organs (Engelhard et al., 1994; Keddie et al., 1989). Resistance to baculovirus infection in some species is attributed to haemocytes avoiding infection, and also exhibiting apoptosis-like behaviour (Clarke & Clem, 2002; Feng et al., 2007; Trudeau et al., 2001).

In the context of mosquito-borne viruses, the route of virus dissemination is not well understood. Work with vector–virus systems including *Aedes albopictus*–Sindbis virus (SINV), *Aedes aegypti*–Dengue virus (DENV), *Ochlerotatus taeniorhynchus*–Venezuelan equine encephalitis virus and *Culex pipiens* pipiens–Rift Valley fever virus indicates the presence of viral antigen in tracheoles, which might shuttle the virus across the midgut basement membrane to initiate dissemination (Bowers et al., 2003; Romoser et al., 2004; Salazar et al., 2007). Midgut muscle tissue is proposed also as a means of amplification and dissemination for West Nile virus (WNV) (Girard et al., 2004).

Whether via the tracheoles or muscle tissue, mosquito-borne arboviruses inevitably encounter the haemocoel environment and haemocytes en route to the salivary glands. In response to pathogens, mosquito haemocytes phagocytose bacteria, yeast and *Plasmodium* parasites; mediate melanization of some nematode parasites, yeast and bacteria; and are involved in cell signalling events for the production of antimicrobial peptides together with the fat body (Bartholomay et al., 2004; Hernandez-Martinez et al., 2002; Hillyer et al., 2003a, b, 2004; Infanger et al., 2004; Lowenberger, 2001). The role of mosquito haemocytes in arbovirus replication, dissemination and antiviral responses is unknown. Infection of mosquito haemocytes with an arbovirus has been noted, but not characterized to date (Foy et al., 2004; Salazar et al., 2007; Sriurairatna & Bhamarapravati, 1977), which is not surprising, because these cells are few and difficult to collect (Castillo et al., 2006; Hillyer & Christensen, 2002).

Because haemocytes are critical to the outcome of baculovirus infections in lepidopteran larvae, and mosquito-borne arboviruses must traverse the haemocoel to get to the salivary glands, and haemocytes in the haemocoel respond to a variety of pathogens, we hypothesized that haemocytes respond to virus present in mosquito haemolymph. In order to explore the potential role of haemocytes in the amplification and dissemination, or mitigation, of arbovirus infection, we observed haemocytes after infecting mosquitoes with SINV (family Togaviridae, genus Alphavirus) transducing viruses designed to drive production of green fluorescent protein (GFP) (see Pierro et al., 2003).
Mosquitoes were infected with SINV TE/5’2J/GFP or MRE16-eGFP via intrathoracic injection. Intrathoracic injection is a standard procedure for delivering a consistent dose of virus to each experimental mosquito (see Bowers et al., 1995, 2003). Also, for comparative purposes, intrathoracic injection was the only method by which mosquitoes could be infected with both TE/5’2J/GFP and MRE16-eGFP because TE/5’2J/GFP is not infectious per os (Foy et al., 2004; Pierro et al., 2003). However, it should be noted that needle injection elicits a rapid cellular and humoral immune response in mosquitoes (Lai et al., 2001) and is not a natural route of arbovirus infection. Both TE/5’2J/GFP and MRE16-eGFP were produced using standard techniques (see Olson et al., 2000) and a volume of 0.5 μl of 1×10⁸ p.f.u. ml⁻¹ was injected per mosquito in all experiments. Haemolymph was collected by perfusion onto microscope slides.

Initial experiments were done with Ae. aegypti (Liverpool strain), a primary vector of Yellow fever and DENV viruses. Haemocyte infection was observed in three separate experiments at 6, 12, 18, 24, 36, 48, 60, 72, 84 and 96 h post injection (p.i.) using fluorescence and phase microscopy. Haemocytes from TE/5’2J/GFP infected mosquitoes exhibit GFP expression as early as 6 h p.i. and for 4 days p.i. (Fig. 1). Although oenocytoids sometimes appear infected, granulocytes are the haemocyte type most often infected with virus. Granulocytes are the most abundant circulating haemocyte type; these phagocytic cells attach to glass surfaces and are easily distinguished by pseudopodia (Castillo et al., 2006; Hillyer & Christensen, 2002). At 48 h p.i. and at every time point thereafter, virus-infected cells exhibit bulbous extensions of the cytoplasm and plasma membrane. At 84 h p.i., the intensity of GFP fluorescence and number of infected granulocytes begins to decline. This does not appear to be a result of cell death, because total haemocyte numbers do not decrease.

Using one cohort of mosquitoes, infected haemocytes were observed and counted based on the presence or absence of GFP fluorescence. The proportion of infected cells throughout the time-course is as follows: 45% (6 h p.i.), 88% (12 h p.i.), 96% (18 h p.i.), 100% (24 h p.i.), 89%
Fig. 2. Replication of SINV TE/5’2J/GFP in infected Ae. aegypti was measured by TCID50 using Vero-76 cells, at various time points post intrathoracic injection from approximately eight drops of dilute haemolymph. Each data point is an average value from five mosquitoes. Virus replication in the haemolymph is significantly higher at 24, 36, 48 and 60 h p.i. as compared with earlier and later time points. Statistical significance was calculated using the Tukey–Kramer HSD test, displayed as letters above the error bars. Values that are not represented by the same letter are significantly different.

(36 h p.i.), 87% (48 h p.i.), 83% (60 h p.i.), 86% (72 h p.i.), 75% (84 h p.i.) and 70% (96 h p.i.).

In keeping with previous reports on TE/5’2J/GFP where virus was first observed 3 days p.i. (Pierro et al., 2003, 2007), virus is first observed in head squashes at 60 h p.i. in 87.5% (7/8) of Ae. aegypti. At all later time points (72, 84 and 96 h p.i.), 100% (10/10, 6/6 and 7/7 mosquitoes, respectively) of head tissues are virus infected.

GFP expression and accumulation is a valid measure of SIN-V-GFP replication (Foy et al., 2004; Olson et al., 2000; Pierro et al., 2003). However, to further confirm that virus replication takes place in haemocytes, the titre of TE/5’2J/ GFP in Ae. aegypti haemolymph was calculated using an end-point dilution assay at different time points post injection, and expressed as TCID50 (Fig. 2). Haemolymph from saline-injected mosquitoes was used as a negative control and did not cause cytopathic effects. Virus titres from mosquito haemolymph are lowest at 6 h p.i., and increase over time. Titres from haemolymph collected at 24, 36, 48 and 60 h p.i. are significantly higher than those at earlier (6, 12 and 18 h p.i.) or later (72, 84 and 96 h p.i.) time points. The highest titre log TCID50 ml⁻¹ (9.77 ± 0.52) is observed at 48 h p.i. in the haemolymph of infected mosquitoes.

The increase in virus titre observed in haemolymph samples is likely solely attributed to virus replication in haemocytes because (i) the tissue tropisms for this virus are limited (Pierro et al., 2007), (ii) infection is not evident during the observation period in the midgut, Malphigian tubules and ovaries in this (data not shown) and a previous study (Olson et al., 1996), and (iii) virus amplification is not evident in head tissue until 60 h p.i., 26 h after a significant increase in haemolymph virus titre occurs. Furthermore, in two previous studies in which SINV was used to silence phenol oxidase (PO), PO suppression was evident for 15 days p.i. (Shiao et al., 2001; Tamang et al., 2004). Because these viruses produce the gene of interest from a subgenomic promoter, antisense PO production necessitates virus replication, and because PO is a haemocyte-specific gene, virus replication had to occur in haemocytes.

To investigate whether the observed phenomenon was species specific, haemocytes from additional species of mosquitoes (Aedes triseriatus, Ae. albopictus and Culex pipiens) were observed after injection with TE/5’2J/GFP. Experiments were done with at least three different cohorts of mosquitoes. Haemocytes were checked for GFP expression at 24 and 48 h p.i., when the highest percentage of virus-infected haemocytes is observed in Ae. aegypti. In all three species, virus-infected haemocytes are evident at 24 and 48 h p.i. (Fig. 3a–l). The proportion of haemocytes infected with the virus is as follows at 24 and 48 h p.i.: 69% and 76% (Ae. triseriatus), 72% and 65% (Ae. albopictus), 53% and 65% (Cx. pipiens). As seen with Ae. aegypti, granulocytes exhibit the most GFP fluorescence in TE/5’2J/ GFP-infected Ae. triseriatus, Ae. albopictus and Cx. pipiens.

Cx. pipiens exhibit fewer fluorescent haemocytes, and GFP fluorescence intensity is lower than that of Aedes species granulocytes, but the proportion of infected haemocytes is not significantly different according to Tukey–Kramer HSD analysis (data not shown). In another study of Culex–SINV tissue tropisms, midguts were almost completely resistant to oral infection (Foy et al., 2004).

Infection of the head tissue of Ae. triseriatus, Ae. albopictus and Cx. pipiens is detected at 48 h p.i. in 88.8% (8/9) of mosquitoes of all three species, earlier than was observed in the head tissue of infected Ae. aegypti.

SINV MRE16-eGFP was injected into the haemocoeel of Ae. aegypti mosquitoes to investigate whether haemocytes can be infected with a virus that has the full complement of genes required to infect and disseminate from the midgut (see Foy et al., 2004; Myles et al., 2003). Haemocytes from Ae. aegypti injected with MRE16-eGFP exhibit fluorescence at 24, 48, 72 and 96 h p.i. (Fig. 3m–p). The number of SINV MRE16-eGFP-infected haemocytes does not change significantly over time during this period. Granulocytes are the cell type most frequently infected with MRE16-eGFP.

Infection of Ae. aegypti head tissue with the MRE16-eGFP virus was assayed in head squashes in three separate experiments. Infection of head tissue is never observed at 24 h p.i. (0/12 mosquitoes tested). At 48 h p.i., 33% (5/15) and at 72 h p.i., 87% (13/15) of mosquitoes have evidence of GFP expression in the head. At 96 h p.i., 100% (12/12) of mosquitoes tested have infected head tissue.
Mosquito haemolymph is suspected to be critical for systemic arbovirus infection (see Foy et al., 2004); however, it is generally believed that virions exist in a free state within the haemolymph. The results presented here show that haemocytes in the haemolymph can be infected with two different SINVs, in multiple species of mosquito. Moreover, our results show that this arbovirus can exploit haemocytes as a site for replication, rather than just existing free in the haemolymph. Since WNV takes 5–8 days to reach the salivary glands after dissemination from the midgut, Girard et al. (2004) speculated that some arboviruses may require an amplification site outside the midgut epithelium. Furthermore, Hardy et al. (1983) speculated that low concentrations of virus in a viraemic blood meal would necessitate a secondary amplification site other than the midgut epithelium. Based on this work with SINV, it becomes clear that haemocytes can function in virus incubation and replication, and so are a strong candidate site for arbovirus amplification en route to the salivary glands.

These data, together with previous studies indicating the involvement of tracheae in disseminating arboviruses (Romoser et al., 2004; Salazar et al., 2007), support an emerging model for dissemination of arbovirus, similar to the current model for baculovirus dissemination in lepidopteran larvae, where the virus disseminates from the midgut to the tracheoles and then into haemocytes that are critical for systemic infection (Engelhard et al., 1994; Keddie et al., 1989). Since haemocytes are critical in the mosquito innate immune response, they could also serve as a barrier to arbovirus infection and dissemination. Documented barriers to infection and dissemination in a mosquito host include the midgut infection barrier, midgut escape barrier, salivary gland infection barrier (SIB) and salivary gland escape barrier (see Black et al., 2002). Overcoming these barriers is critical for an arbovirus to successfully replicate and disseminate, and to be transmitted to the next vertebrate host. Hardy et al. (1983) speculated that haemolymph could be an important determinant of the SIB because virus titres in the haemolymph of Cx. tarsalis susceptible to Western equine encephalitis virus were significantly higher than in those of refractory females. This is the first study to demonstrate that mosquito haemocytes are a cell type in which arbovirus infection and replication occurs. Further studies of haemocyte infection post exposure to an arbovirus via blood feeding are necessary. However, the implications of these initial findings are significant because, depending on the vector–virus system, these cells could be a site of virus amplification, serve as a conduit for virus dissemination, or present a barrier to further dissemination.

**Acknowledgements**

We are grateful to Drs Ken Olson and Dennis Pierro (Colorado State University, CO, USA) for plasmids and technical assistance. Thanks
to Huarong Li for help in virus preparation, to Jessica Petersen for assistance with statistical analysis, to Brianne Simonsen for mosquito rearing, and to Brad Tucker for technical assistance (all Iowa State University, IA, USA). Support for this study was provided by The Center for Integrated Animal Genomics, Iowa State University, IA, USA. This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, USA, Project 5111, is based upon work supported by the Hatch Act and State of Iowa funds.

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