The Sigma Viruses of *Drosophila*

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Abstract

The sigma virus of *Drosophila melanogaster* (DMelSV) was discovered accidentally over 70 years ago owing to it causing infected flies to become paralysed and die on exposure to CO_2. Recently, five other species of *Drosophila* and a species of Muscidae fly have also been found to be infected with sigma viruses, and together these viruses form a major clade of rhabdoviruses that is a sister clade to the dimarhabdoviruses. In those cases where the transmission of these viruses has been investigated, sigma viruses are transmitted purely vertically through infected eggs or sperm. In natural populations of insects the spread of these viruses can be very rapid, and has led to flies evolving resistance to sigma viruses. Two resistance genes have been identified in *D. melanogaster*, one of which is involved in autophagy.

Discovery of the sigma virus of *Drosophila melanogaster* (DMelSV)

The sigma virus of *Drosophila melanogaster* (DMelSV) was discovered by chance in 1937, when French researchers found that some flies became paralysed and died when exposed to CO_2 during routine anesthetization, rather than recovering as normal (L'Heritier and Teissier, 1937). They found that the trait was transmitted vertically from parent to offspring, but was independent of the host chromosomes. It could also be transferred by injecting haemolymph from flies that were sensitive to CO_2 into non-sensitive flies, suggesting that the trait was caused by an infectious agent. The trait was later attributed to the presence of a virus-sized particle, which they named sigma (L'Heritier, 1948). The bullet-shaped morphology, antigenic profile and partial genome sequences subsequently identified DMelSV as a rhabdovirus (Berkalof *et al.*, 1965; Teninges, 1968; Calisher *et al.*, 1989; Teninges *et al.*, 1993).

The sigma virus clade

D. melanogaster is not the only species to be infected with a sigma virus. By screening species for CO_2 sensitivity, six additional species of flies were found to be infected with sigma viruses (Longdon *et al.*, 2010, 2011c). These viruses infect five species of *Drosophila* – *D. affinis*, *D. obscura*, *D. tristis*, *D. immigrans* and *D. ananassae* – and one member of the Muscidae, *Muscina stabulans*. These viruses have been tentatively named as DAffSV, DObsSV, DTriSV, DImmSV, DAnaSV and MStaSV, respectively (using the standard *Drosophila* species name abbreviation and SV for sigma virus). As these new viruses have been only recently discovered we know very little about their biology, so the majority of this chapter describes the sigma virus of *D. melanogaster* (DMelSV).

Together the sigma viruses form a clade of viruses that infect *Diptera* (flies), that are closely related to the dimarhabdoviruses (Bourhy *et al.*, 2005); a clade containing the *Ephemerovirus* and *Vesiculovirus* clades of rhabdoviruses (Fig. 8.1 and Fig. 3.2) (Longdon *et al.*, 2010, 2011c). The sigma virus clade contains a greater amount of genetic divergence between its members than seen in four out of the six accepted rhabdovirus genera, suggesting that they are a diverse and major group of

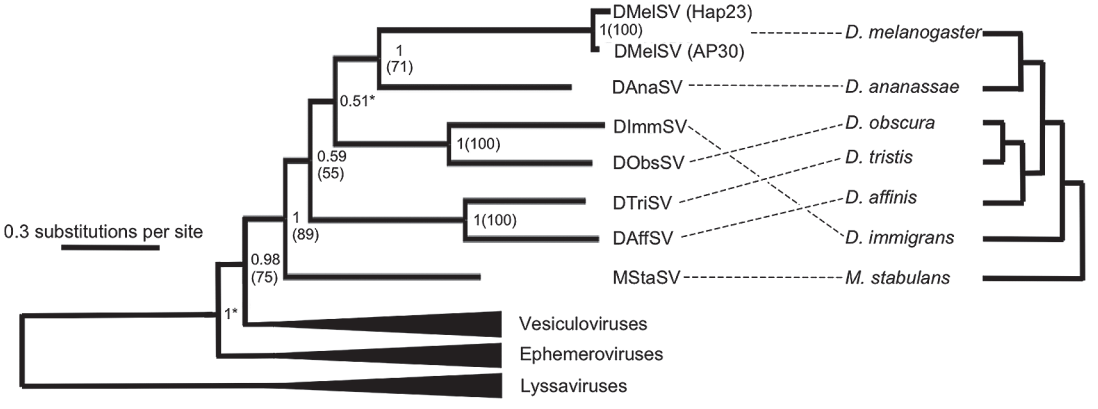

Figure 8.1 Phylogeny of the sigma viruses (left) and their hosts (right), with incongruence between host and virus phylogenies visualized. Node labels represent Bayesian posterior supports with maximum likelihood bootstrap support in brackets. The tree is rooted with the *Lyssavirus* clade. Non-sigma virus clades are collapsed. Nodes marked with an asterisk had bootstrap support of <50%. Figure is reproduced from Longdon *et al*. (2011c).

rhabdoviruses (Longdon *et al.*, 2010). The three viruses (DMelSV, DAffSV and DObsSV) whose transmission mode has been investigated have been shown to be vertically transmitted (Brun and Plus, 1980; Longdon *et al.*, 2011b), suggesting that it is likely that the other sigma viruses are also vertically transmitted.

It is likely that sigma viruses infect a wide range of dipterans if not insects as a whole. Populations of thirteen other species of *Drosophila* (Brun and Plus, 1980) and *Culex quinquefasciatus* mosquitoes (Shroyer and Rosen, 1983) have all been found to contain individuals that are paralysed or killed by CO_2. Furthermore, in *Drosophila athabasca* and *C. quinquefasciatus*, the CO_2 sensitivity is inherited independently of the host chromosomes in a biparental manner similar to that of DMelSV (Williamson, 1959, 1961; Shroyer and Rosen, 1983). As CO_2 sensitivity is a common trait of many insect rhabdoviruses (Bussereau and Contamine, 1980; Rosen, 1980; Shroyer and Rosen, 1983; Sylvester and Richardson, 1992), it seems likely rhabdoviruses, and perhaps sigma viruses, infect a range of different dipterans. Additionally, rhabdovirus sequences have inserted into the genomes of various arthropod species (Katzourakis and Gifford, 2010) and rhabdovirus-like particles have been found in firebug testes (Afzelius *et al.*, 1989). Recently a new rhabdovirus has been found in *Culex tritaeniorhynchus*, which has been suggested to cluster within the sigma virus clade (Kuwata *et al.*, 2011). However, this may be an artefact of the method used to infer the phylogeny (B. Longdon, unpublished data) and the virus actually represents a distinct lineage that sits as an out-group to the sigma and dimarhabdovirus clades. Nevertheless, this and the previous results highlight that there are likely many more sigma-like viruses, and other rhabdoviruses, to be discovered in invertebrates.

The discovery of a sigma virus outside the genus *Drosophila* (in *M. stabulans*) is of particular interest as the closely related dimarhabdoviruses are vector-borne pathogens of vertebrates, some of which are vectored by other dipterans (Dacheux *et al.*, 2010). It is currently impossible to distinguish whether the ancestor of the sigma viruses and dimarhabdoviruses evolved from vertebrate-infecting viruses that switched to being purely entomopathogenic in the sigma virus clade, or whether the ancestors of the dimarhabdoviruses were viruses of arthropods that came to infect vertebrates.

Genetics

The genome organization of DMelSV is similar to that of other rhabdoviruses, with 3′-N–P–M–G–L-5′ genes, but with an additional gene of unknown function (the X gene) located

between the P and M genes (Teninges *et al.*, 1993; Contamine and Gaumer, 2008; Longdon *et al.*, 2010). The X gene (sometimes referred to as gene 3) shares structural similarities to viral RNA polymerases. It is predicted to have an N-terminal signal peptide and has three predicted N-glycosylation sites suggesting it may be a secreted glycoprotein (Landesdevauchelle *et al.*, 1995; Longdon *et al.*, 2010; Walker *et al.*, 2011). Some other rhabdoviruses also contain one or more accessory genes (Walker *et al.*, 2011); the plant-arthropod infecting rhabdoviruses also have an additional gene located between the P and the M genes (Ammar *et al.*, 2009) and some dimarhabdoviruses also contain additional genes (Gubala *et al.*, 2008, 2010; Zhu *et al.*, 2011). In total the DMelSV genome is 12,625 nucleotides (nt) long. Following each gene are motifs with seven U residues (3′-GUACUUUUUUU-5′), which are thought to be transcription termination sequences triggering polyadenylation of the mRNA (Teninges *et al.*, 1993). In DMelSV the putative transcription initiation site has been identified as 3′-GUUGUNG-5′ for all genes except the N gene, where it is 3′UUGUUG-5′ (Teninges *et al.*, 1993). The only other sigma virus with a near complete genome sequence is DObsSV which has the same genome organization as DMelSV, and is 12,676 nt long (excluding the 5′ trailer) (Longdon *et al.*, 2010). The full length L gene has been sequenced for DAffSV, and other sigma viruses have partial L gene sequences available (Longdon *et al.*, 2010; Longdon *et al.*, 2011c). The viral substitution rate has been estimated (Carpenter *et al.*, 2007) by sequencing a DMelSV strain that had been split and maintained in two different *Drosophila* strains for 10–20 years. It was estimated that there were 4.6×10^{-5} substitutions/site/year, which is equivalent to approximately 0.6 substitutions per genome per year. The laboratory-derived mutation rate was not found to be significantly different from that of isolates collected in the field (Carpenter *et al.*, 2007).

Recombination is very rare in negative-sense RNA viruses *per se* (Chare *et al.*, 2003) but is perhaps even less likely in sigma viruses where the chance of multiple infections is low as this would require both parent's viral type to infect the offspring (see below). Despite early suggestions that DMelSV might recombine (Ohanessian-Guillemain, 1959), statistical analyses of sigma virus RNA sequences (DMelSV and DObsSV) from the field have found that recombination is very rare or absent (Carpenter *et al.*, 2007; Longdon *et al.*, 2011b; L. Wilfert, unpublished data).

Mode of natural transmission

DMelSV is transmitted vertically through both sperm and eggs with horizontal transmission being rare or absent (L'Heritier 1957; Brun and Plus, 1980; Fleuriet, 1988). This is a rather unusual mode of transmission, as although numerous vertically transmitted pathogens are known from insects, most of these are only transmitted through eggs, or they are also transmitted horizontally (Buchner, 1965; Engelstadter and Hurst, 2009). How males transmit the virus is unclear, but viral particles have been observed in sperm cells (Teninges, 1968). This biparental mode of transmission is essential for the virus to spread through the host population, as a purely maternally transmitted parasite will go extinct, even if transmitted perfectly, if it is costly to its host. It has been shown that by transmission through both males and females, parasites can persist and spread through host populations even if infected hosts suffer a fitness cost (L'Heritier, 1970; Fine, 1975).

The rate of DMelSV transmission differs between the sexes (Fig. 8.2) (L'Heritier, 1948, 1957, 1970; Fleuriet, 1988). Firstly, females transmit the virus at a higher rate than males. Secondly, the transmission rate of a fly is reduced when it is infected by its father rather than its mother. Females inheriting the virus from their mother are termed 'stabilized', as their rate of transmission is close to 100%. However, if a female is infected by her father (termed 'unstabilized'), her average transmission drops to a much lower rate of about 80% (Fleuriet, 1988). Similarly, while a 'stabilized' male infected by his mother can transmit the virus to his offspring (albeit at a lower rate than females), a male that is infected by his father does not transmit the virus at all. Therefore, it seems the virus cannot be transmitted through males for two successive generations.

The lower rates of transmission in flies infected

from their father rather than their mother seems to have a physiological cause, as sperm transfer a lower viral titre to the developing embryo (Plus, 1955; L'Heritier, 1957, 1970). This is thought to lead to a failure to infect the early-stage germ line, and the gametes may never become infected later in development, even if the viral titres recover to higher levels in other tissues later in life (Plus, 1955; L'Heritier, 1957, 1970). Further evidence for the difference in viral titres transmitted in sperm and eggs comes from crosses when both parents are infected, which results in the maternal viral type being inherited in the vast majority of cases (Ohanessian-Guillemain, 1959). This is consistent with the observation that few wild caught flies show evidence of multiple infections (Longdon *et al.*, 2011b).

Other factors can also affect the rate of transmission. Females with high ovarian viral titres have high rates of transmission (Bregliano, 1970; Brun and Plus, 1980). After a female has mated with an infected male, the proportion of infected offspring declines over time, which could suggest infected sperm have reduced longevity or the virus is lost from sperm over time (Plus, 1955; L'Heritier, 1957). Transmission of most DMelSV viral strains can also be prevented by placing infected flies at 30°C, and transmission will resume if the parents are returned to lower temperatures (20°C).

Interestingly, viral titres can be about 3–5 times greater in flies infected by their fathers rather than mothers (L'Heritier, 1957, 1970) despite these flies transmitting the virus at a lower rate (Plus, 1955; L'Heritier, 1957). A similar effect is seen after artificial inoculation of the virus, which results in high viral titres but low transmission rates (see Fig. 1 in L'Heritier, 1970). It has been suggested that this may be a viral response to failure to invade the zygote's pole cells (L'Heritier, 1970), causing over-replication in the host in an 'attempt' to get into the germline (L'Heritier, 1970). As evidence for this, in stabilized flies about one half of the total viral load is found in the ovarian cysts (clusters of germ line cells) of egg laying females (L'Heritier, 1970), whereas in non-stabilized females only a few cysts harbour similar viral titres. This means that the majority of the viral load in non-stabilized flies is in somatic tissues, which may represent a failure to invade and replicate to high levels in germ line cells.

Similar patterns of transmission have been observed for DAffSV and DObsSV. While in *D. affinis* males have a lower rate of transmission than females (45% compared with 98%), this is not the case for *D. obscura*, where males have comparable transmission rates to females (92% and 88% respectively) (Longdon *et al.*, 2011b). However, in both cases males infected by their fathers do not transmit the virus. Embryos infected by their fathers (i.e. when the virus is transmitted through sperm rather than eggs) have lower titres of both DAffSV and DObsSV, as has been observed for DMelSV (Brun and Plus, 1980). This likely explains why transmission through sperm is less efficient.

Viral replication following experimental injection

Sigma viruses can be transmitted between individuals in the laboratory by injecting a crude virus suspension (infected adult flies homogenized in Ringer's solution (Sullivan *et al.*, 2000) and briefly centrifuged to remove debris). Following injection, flies that are successfully infected transmit the virus as if they had been infected by their father, with only females transmitting the virus to offspring (L'Heritier, 1957, 1970).

The onset of CO_2 sensitivity following injection depends on the initial dose of the virus, and can be around 5–20 days at 20°C (Brun and Plus, 1980). Following injection with the virus the number of infectious units in a fly increases until about 10–15 days post injection where it plateaus (Brun and Plus, 1980). In females, from around day 10 onwards transmission to offspring can occur in some cases, increasing until around day 20 where it begins to decline (Brun and Plus, 1980). Classically, 'infectious units' were ascertained from taking the homogenate of an infected fly, creating a dilution series and injecting it into non-infected individuals (Plus, 1955; L'Heritier, 1957).

Cross-species infections

Although DMelSV is restricted to *D. melanogaster* by virtue of its vertical transmission, it can replicate

in a range of different dipterans, but not different orders of insects (Jousset, 1969). DMelSV has also been crossed into hybrid offspring with the closely related *D. simulans* (Kalmus, 1943). DMelSV injected into *D. funebris, D. willistoni, D. prosaltans* and *D. gibberosa* causes CO_2 sensitivity. In the two species examined stabilized transmission of injected DMelSV was found to occur in the closely related *D. simulans* but not the more distantly related *D. funebris* (L'Heritier, 1957).

Despite the fact that they appear to be mostly vertically transmitted, there is significant incongruence between the host and sigma virus phylogenies, which indicates that these viruses have switched between different host species during their evolutionary history (Fig. 8.1) (Longdon *et al.*, 2011c). Although there is clear evidence of host switches, there is tentative evidence that the phylogenies of the host and virus are more similar than expected by chance (Longdon *et al.*, 2011c), although more data are required to confirm this finding. This may be due to co-speciation of the virus with the host or may be explained by host shifts more frequently occurring between closely related species. It has also been suggested that parasitic mites could play a role in vectoring the virus between host species (Longdon *et al.*, 2010).

It has recently been found that most of the variation in the ability of three sigma viruses (DMelSV, DAffSV and DObsSV) to persist and replicate in a novel host species is explained by the host phylogeny (Longdon *et al.*, 2011a). From cross- inoculating these viruses into 51 different species of Drosophilidae, it was found that species more closely related to the natural host species had greater viral titres than more distantly related species. Additionally, there is a strong effect of the host phylogeny that is independent of the effect of distance from the natural host, with groups of related species having similar viral titres. This phylogenetic effect is mostly caused by variation in susceptibility to all three viruses (there is a strong phylogenetic correlation in the titres of the three viruses). Such patterns may result from the common ancestors of different host clades having acquired or lost immune or cellular components that affect general susceptibility to all sigma viruses (Jiggins and Kim, 2005; Sackton *et al.*, 2007; Havard *et al.*, 2009; Obbard *et al.*, 2009).

CO_2 paralysis symptom

Sigma virus infection causes flies to become paralysed and die after exposure to CO_2, whilst uninfected flies recover after a short period (L'Heritier and Teissier, 1937). In DMelSV the paralysis seems to be due to damage caused to nervous tissue, in particular the thoracic ganglia, with flies being injected with the virus at a site close to the thoracic ganglia developing CO_2 sensitivity in about 2 days compared with the 5- to 10-day delay observed if injected elsewhere in the thorax or abdomen (L'Heritier, 1948; Busserea, 1970a,b). Other rhabdoviruses are known to replicate in nervous tissue, with the glycoprotein (G) of rabies virus preferentially attaching to several neural cell receptors (Lentz *et al.*, 1982; Gastka *et al.*, 1996; Thoulouze *et al.*, 1998; Tuffereau *et al.*, 1998, 2001; Lyles and Rupprecht, 2007), suggesting this may be a general rhabdovirus trait. Furthermore, other rhabdoviruses injected into *Drosophila* (and other insects) also cause CO_2 paralysis (Bussereau and Contamine, 1980; Rosen, 1980; Shroyer and Rosen, 1983) and it has been noted that rhabdovirus infected aphids have reduced longevity after CO_2 exposure (Sylvester and Richardson, 1992). Recent studies have confirmed DMelSV is found at high titres in nervous tissues using immunofluorescent microscopy (Tsai *et al.*, 2008; Ammar *et al.*, 2009).

The CO_2 paralysis in sigma virus infected *D. melanogaster* is brought on by relatively short exposures to CO_2 (< 1 min) and is specific to CO_2, with other gases and injected chemicals having no effect (L'Heritier, 1948; Brun and Plus, 1980). The paralysis is also sensitive to changes in CO_2 concentration and temperature, with lower temperatures requiring lower CO_2 concentrations to cause paralysis, perhaps due to greater gas solubility at low temperatures. At close to 0°C low concentrations of CO_2 are sufficient (< 20%), increasing to 50% at 10°C, with no CO_2 induced paralysis observed above 23°C (Brun and Plus, 1980). The standard assay for infection is exposure

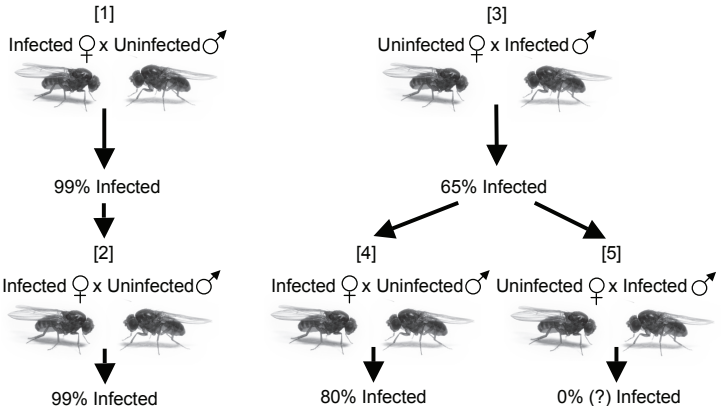

Figure 8.2 Patterns of sigma virus transmission. The rates of transmission are based on DMelSV (Fleuriet, 1988), but patterns are similar for DAffSV and DObsSV (Longdon *et al*., 2011b) (although see main text for minor differences with DObSV). Infected females crossed to uninfected males (1) transmit the virus to close to 100% of their offspring (range of 98–100%), and are referred to as 'stabilized'. If daughters from this cross are again crossed to uninfected males (2) they too will transmit to close to 100% of their offspring. If infected males are crossed to uninfected females (3) they will transmit the virus to approximately 65% of their offspring on average (with a range of 0–100%). If infected daughters from this cross are mated to uninfected males (4) they will transmit the virus at a lower rate than daughters infected by their mothers (about 80% on average, with a range of 0–100%), and are referred to as 'unstabilized'. If infected sons from (3) are crossed to uninfected females (5) they do not transmit the virus at all, suggesting that the virus transmission through two successive male generations is rare or absent. Fly photo credit: Darren Obbard and Ben Longdon.

at 12°C for 15 minutes, with flies assessed for paralysis about 30 minutes later.

Although we know the thoracic ganglion is involved in CO_2-induced paralysis (Brun and Plus, 1980) the exact mechanism is unknown. Interestingly, CO_2 exposure will lower the pH of flies' haemolymph, and both vesicular stomatitis virus (VSV) and rabies show enhanced binding at reduced pH. Furthermore, rhabdovirus G proteins induce membrane fusion in a pH-dependent manner, suggesting a potential mechanism (Lyles and Rupprecht, 2007). However, crude attempts to manipulate fly pH by injecting acidic solutions (hydrogen cyanide, formic acid and hydrochloric acid) were found to have no effect on DMelSV infection (L'Heritier, 1948; Brun and Plus, 1980). Additionally, rhabdoviruses bind to acetylcholine receptors on nervous tissue that could also be involved in paralysis but assays of acetylcholinesterase activity, which degrades acetylcholine at synapses, found no difference between infected and uninfected flies (Brun and Plus, 1980). Furthermore, a dose of CO_2 below the threshold required to cause paralysis can protect infected *D. melanogaster* from a subsequent dose that would normally be lethal (L'Heritier, 1948). While injection of infected fly haemolymph into uninfected flies will cause CO_2 sensitivity about 10–15 days post inoculation, there is no immediate effect. This suggests that the effect is not due to mere physiological changes in the haemolymph but is induced by viral replication (L'Heritier, 1970).

It is unclear if the CO_2 sensitivity has any relevance in nature, although rotting fruit or plants, which are often *Drosophila* oviposition sites (Basden, 1954), will produce CO_2. This could have potentially sublethal effects on flies in enclosed spaces at low temperatures, under which conditions a lower concentration of CO_2 is required for paralysis (Brun and Plus, 1980). The effect of CO_2 is not limited to adults, but also causes death in larvae, although early stage embryos and pupae do not seem to be affected (L'Heritier, 1948). CO_2 paralysis has been clearly demonstrated in *D. melanogaster* and is tightly linked to sigma virus infection (Brun and Plus, 1980; Wilfert and Jiggins, 2010b), but can be less clear-cut in other species (B. Longdon personal observation), and some infected *D. obscura* do not display CO_2 paralysis (Longdon *et al*., 2011b).

Costs of sigma virus infection to the host

Vertically transmitted pathogens rely on their hosts surviving and reproducing to be transmitted, and therefore tend to cause little harm to their hosts (i.e. they have a low virulence) (Fine, 1975). This appears to be the case with DMelSV, as infected fly stocks are generally healthy in the laboratory, but nonetheless experimentation has shown that the virus does cause some harm to the *Drosophila* host. DMelSV rapidly spreads through laboratory populations of flies kept at a low density, but if these populations were placed at high densities – so the flies have to compete for resources – the virus showed a decline in frequency (Yampolsky *et al.*, 1999). This can be explained by the virus reducing the fitness of infected flies, so they are out-competed and therefore unable to pass the infection on to future generations. Given that the virus is usually only found at low prevalence in the field (0–20%) (Fleuriet, 1988; Carpenter *et al.*, 2007; Wilfert and Jiggins, 2010a), it is likely that DMelSV is causing a fitness cost to wild flies as well. Based on the rate of expansion in lab populations, the reduction in fitness in the field has been estimated at 20–30% (Yampolsky *et al.*, 1999).

Several studies have examined the physiological basis of this fitness cost. Infected flies have reduced egg viability in the laboratory, (Seecof, 1964; Fleuriet, 1981a) and may take longer to develop from egg to adult by about 5–10 hours (Seecof, 1964). Seecof (1964) found a 20% reduction in egg viability in infected flies, but used only a single fly line. Fleuriet (1981a) used multiple fly lines and her data suggest an overall reduction in egg viability of about 10% in infected lines (averaging each line over all crosses, exact Wilcoxon rank sum test: $P = 0.049$, following re-analysis by B. Longdon). Similarly, a reduction in female fertility has been reported for another viral isolate, yet this was not due to a reduction in egg viability but because the ovarian cysts of infected individuals developed slower than uninfected ones (Jupin *et al.*, 1968). Additionally, a study under semi-natural conditions reported that virus-infected flies declined in number if over-wintered as adults (Fleuriet, 1981b). However, the patterns observed were not clear, with only five out of the six populations tested showing this trend, but even then the pattern was subjective (Fleuriet, 1981b). Studies of CO_2 sensitivity in wild populations of *D. melanogaster* show some evidence of declines in infection after winter, but the data are inadequate to be conclusive (Herforth and Westphal, 1966; Felix *et al.*, 1977). Additionally, it has been reported that DMelSV reduces flies' resistance to the fungal pathogen *Beauvaria bassiana* in a laboratory experiment (about 10% increase in mortality in DMelSV-infected flies exposed to fungi), although no suppression of the Toll pathway (which is known to be involved in anti-fungal resistance) was observed (Carpenter, 2008).

Host resistance

Given the fitness cost of DMelSV to its host, it is not surprising that *D. melanogaster* has evolved resistance mechanisms towards the virus. Most of the work on resistance has focussed on naturally occurring genetic polymorphisms that alter the susceptibility of wild flies to DMelSV infection (Gay, 1978; Bangham *et al.*, 2008). More recently, attention has also turned to the question whether there is an induced immune response to sigma virus infection.

Ref(2)P gene

The best-studied polymorphism controlling resistance to DMelSV is in the gene *ref(2)P* (Guillemain, 1953; Contamine *et al.*, 1989). Resistance is due to a mutation in the PB1 domain of the encoded protein that changes the amino acids Gln-Asn to a single Gly (Dru *et al.*, 1993; Wayne *et al.*, 1996; Bangham *et al.*, 2007). When flies are injected with certain strains of DMelSV, they are less likely to be successfully infected if they carry the resistance allele (Fig. 8.3) (Ohanessian-Guillemain, 1963; Carre-Mouka *et al.*, 2007). The polymorphism also has an effect on the rate at which flies transmit the virus to their offspring. The effect is greatest when the virus is transmitted from an infected female through eggs – only 8% of offspring homozygous for the resistance allele became infected compared to 99% of susceptible offspring (Bangham *et al.*, 2008). The resistance allele is associated with a 59% decline in male transmission in homozygous offspring (Bangham *et al.*, 2008).

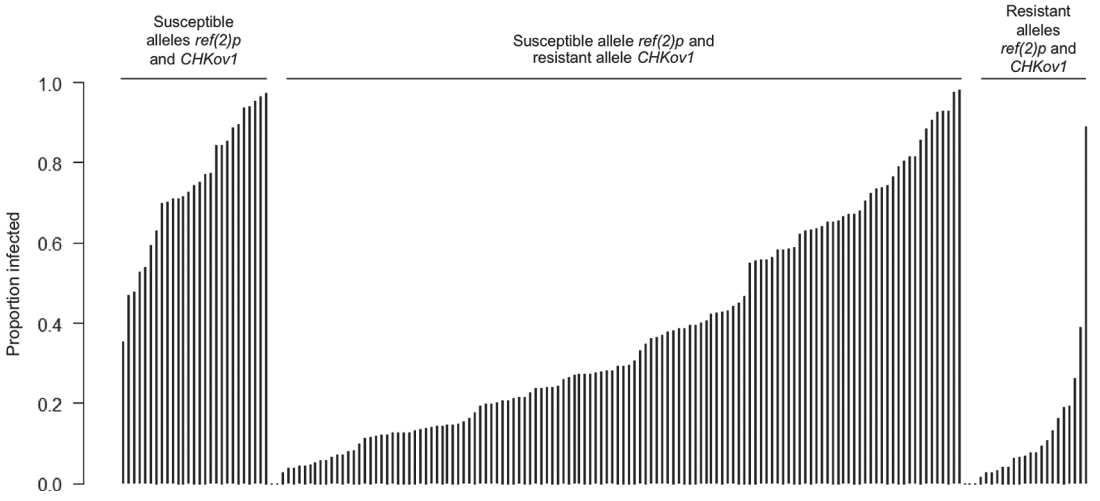

Figure 8.3 The effect of two polymorphic resistance genes on infection rates in 176 inbred lines of *Drosophila melanogaster* (Magwire *et al*., 2011). The flies were injected with DMelSV and classified as infected or uninfected using the CO_2 sensitivity assay. Each bar represents a different inbred fly line that originated from a population in North Carolina. Details of the experiment are described in Magwire *et al*. (2011).

This polymorphism is an important factor in affecting the rate of transmission of the virus in nature. The frequency of the resistant allele can be high, ranging from 24–36% in Europe (Fleuriet and Sperlich, 1992; Wilfert and Jiggins, 2010a). Furthermore, infected female flies in the field transmit the virus to 28% fewer offspring if they carry the resistant allele (Wilfert and Jiggins, 2010a). Being able to replicate laboratory results in the field is a crucial finding if results are to be extrapolated to the real world.

The mechanism by which *ref(2)P* makes flies resistant to sigma virus is unknown. However, it has recently been discovered that this protein plays a key role in autophagy, which is an important defence mechanism against intracellular pathogens in animals (Pankiv *et al*., 2007; Nezis *et al*., 2008; Shelly *et al*., 2009). Autophagy is a process by which components of the cytoplasm are encapsulated inside double membrane vesicles known as autophagosomes, which fuse with lysosomes and are then degraded (Klionsky and Ohsumi, 1999). Ref(2)P, which is called p62 in most other organisms, is an adaptor protein which selectively targets polyubiquitinated substrates for degradation by autophagy (Korolchuk *et al*., 2009; Lamark *et al*., 2009). In *Drosophila*, ref(2)P localizes with poly-ubiquitinated protein aggregates and accumulates in cells when autophagy is inhibited (Nezis *et al*., 2008). The role of autophagy in innate immunity has mainly been investigated as a defence against intracellular bacteria, and here p62 interacts with ubiquitinated proteins on the bacterial surface and is essential for targeting bacteria to the autophagic pathway (Zheng *et al*., 2009). It is now becoming clear that autophagy can also defend cells against viruses (Levine and Deretic, 2007; Shelly *et al*., 2009). In *Drosophila*, VSV activates autophagy, protecting flies against the virus (Shelly *et al*., 2009), while in mammalian cells other viruses are able to evade this form of immune defence by suppressing autophagy (Orvedahl *et al*., 2007; Dreux and Chisari, 2010).

The observation that autophagy can protect flies against VSV suggests a model by which *ref(2)P* protects *Drosophila* against sigma virus infection. Because VSV and the sigma viruses are moderately close relatives (Longdon *et al*., 2010), it is likely that autophagy also plays an antiviral role against the sigma virus. Furthermore, null mutants of *ref(2)P* are susceptible to sigma virus infection, so the resistant allele of this gene appears to have an antiviral effect rather the susceptible allele having a proviral effect (Contamine *et al*., 1989). Co-immunoprecipitation experiments have also shown that the viral P protein interacts

with ref(2)P (Wyers *et al.*, 1993). Therefore, the resistant ref(2)P isoform may protect flies against infection by targeting the sigma virus for degradation by autophagy, possibly by acting as a pattern recognition receptor that binds to viral proteins.

Ref(2)P/p62 is also involved in processes other than autophagy that could explain its role in virus resistance. p62 can target ubiquitinated cargoes for degradation by the proteasome, and this could potentially affect viral replication (Nezis *et al.*, 2008). It is also important in rel/NF-κB immune responses (Avila *et al.*, 2002), but previous work has shown that the Toll signalling pathway does not affect sigma virus titres, so it is unlikely that this is the cause of resistance (Carpenter *et al.*, 2009a).

CHKov genes

A second major-effect polymorphism (previously referred to as *ref(3)D*; Gay, 1978) that makes flies resistant to the sigma virus was recently mapped to a region containing two paralogous genes called *CHKov1* and *CHKov2* (Magwire *et al.*, 2011). It was found that three alleles of these genes occur, which confer different levels of resistance. The ancestral state is for *CHKov1* and *CHKov2* to both be intact genes, and these flies are susceptible to DMelSV. The second allele has a transposable element insertion in the protein coding sequence of *CHKov1,* and this is associated with increased resistance to infection (Fig. 8.3) (Magwire *et al.*, 2011). This insertion results in a truncated messenger RNA that encodes a far shorter protein than the susceptible allele (Aminetzach *et al.*, 2005). The third allele consists of two duplications, resulting in three copies of both the truncated allele of *CHKov1* and *CHKov2* (one of which is also truncated) (Magwire *et al.*, 2011). These flies have even greater resistance than those carrying the transposable element insertion alone. Therefore, two major mutational events that have affected the structure of these genes have led to sequential increases in resistance.

The allele carrying the transposable element (conferring a moderate level of resistance) is the commonest in North American populations, occurring at a frequency of 0.82 in North Carolina, while the most resistant allele carrying the duplications is rare and has only ever been found in a single stock collected in France (Magwire *et al.*, 2011). Analysis of the patterns of genetic variation around the *CHKov* genes has shown that natural selection has caused the resistance allele to rapidly increase in frequency (Aminetzach *et al.*, 2005), providing further evidence of the benefits it provides flies in the wild.

It is well known that pathogen resistance genes often have pleiotropic effects on other traits (Kraaijeveld and Godfray, 1997; McKean *et al.*, 2008). These pleiotropic effects tend to be harmful and it is commonly thought that resistance to pathogens is a costly trait to evolve (Kraaijeveld *et al.*, 2002). However, previous research has found that the transposable element insertion in *CHKov1* increases resistance to organophosphate insecticides (Aminetzach *et al.*, 2005). Therefore, contrary to received wisdom, this resistance allele would appear to be beneficial to flies in other ways beyond making them resistant to DMelSV. Interestingly, population genetic analyses suggest that the transposable element insertion occurred well before the use of insecticides (Aminetzach *et al.*, 2005), so it appears likely that originally this allele played a role in antiviral defence, and then it fortuitously 'preadapted' flies to the use of insecticides (Magwire *et al.*, 2011).

The reasons why these genes affect virus and pesticide resistance are unknown. Neither *CHKov1* nor *CHKov2* are upregulated in infected flies (Carpenter *et al.*, 2009a), so they do not appear to be part of an induced immune response. It has been suggested that CHKov1, which contains a choline kinase domain, might make flies resistant to organophosphates by affecting choline metabolism in general or the target of organophosphate insecticides, acetylcholine esterase (Aminetzach *et al.*, 2005). If this is the case, it is possible that it could be linked to the mechanism of virus resistance as other rhabdoviruses use acetylcholine receptors to enter cells (Lentz *et al.*, 1982; Gastka *et al.*, 1996); however, this remains highly speculative.

Other resistance genes

Although the *CHKov* genes and *ref(2)P* are the only two host resistance polymorphisms that have been identified in *D. melanogaster* at the molecular

level, several more genes remain to be characterized. These include resistance genes that have been mapped that delay the onset of CO_2 sensitivity and reduce the viral titres after flies are injected with the virus (Gay, 1978; Brun and Plus, 1980). However, it seems that resistance may sometimes involve blocking the vertical transmission of the virus without impeding viral replication, as there is little genetic correlation between the rate males transmit the virus and the rate they develop CO_2 sensitivity after they have been injected (in *ref(2) P* susceptible flies) (Bangham *et al.*, 2008). Two unidentified genes, which map to the second and third chromosomes of *D. melanogaster*, have been found to reduce the rate of transmission through sperm (Gay, 1978; Bangham *et al.*, 2008).

Immune activation

Drosophila mounts potent immune responses when infected by fungi, bacteria and viruses (Lemaitre and Hoffmann, 2007; Sabin *et al.*, 2010). RNA silencing, autophagy and inflammatory signalling pathways may contribute to the antiviral immune response against *Drosophila* C virus and several entomopathogenic viruses such as Flock House virus (Sabin *et al.*, 2010). Only two studies have examined the innate immune response to DMelSV, but with conflicting results (Tsai *et al.*, 2008; Carpenter *et al.*, 2009a). Tsai *et al.* (2008) used quantitative RT-PCR to show that the sigma virus upregulated several antimicrobial peptides and two peptidoglycan recognition proteins (PGRPs). However, Carpenter *et al.* (2009a) found that the virus had very little effect on the expression of any immune system genes. The reasons why the results of the two studies differ are unclear. It is possible that only certain sigma virus genotypes activate an immune response in certain fly genotypes or conditions. For example the fly lines may have differed in their sigma virus resistance genes. It can also not entirely be ruled out that some other factor such as secondary infections with bacteria were inducing an immune response in the study of Tsai *et al.* (2008).

There is evidence for an innate immune response against VSV (which is in the sister clade to the sigma viruses; Longdon *et al.*, 2010) when injected into *D. melanogaster* (Shelly *et al.*, 2009; Mueller *et al.*, 2010). Firstly it has recently been found that VSV induces autophagy, as discussed above (Shelly *et al.*, 2009). Secondly, the RNA interference (RNAi) pathway is involved in resistance to VSV in *D. melanogaster*, with mutant flies lacking RNAi components showing increased mortality following VSV infection (Mueller *et al.*, 2010). Antiviral RNAi relies on double-stranded (ds) RNA to generate small interfering RNAs (siRNAs), but negative sense RNA viruses produce very little dsRNA compared to other RNA viruses (Obbard *et al.*, 2009). Nonetheless, while no detectable amounts of ds RNA were found in VSV-infected cells, viral siRNAs were detected (Mueller *et al.*, 2010). These siRNAs equally matched both positive and negative-sense genomic copies of the virus, suggesting that they have come from dsRNA formed between the viral genome and its replication intermediate, rather than dsRNA as a result of secondary structure in the viral genome. However, VSV is normally orally transmitted in its natural dipteran vectors (although see Tesh *et al.*, 1972) and so needs to pass through the gut wall to cause systemic infection, whereas sigma viruses are exclusively vertically transmitted in nature (Brun and Plus, 1980; Longdon *et al.*, 2011b). The immune response to horizontally and vertically transmitted viruses may differ substantially, and it remains to be seen if such induced responses occur in sigma virus-infected flies.

RNA editing

Adenosine deaminases that act on RNA (ADARs) are enzymes that introduce mutations in double stranded RNA, changing adenosine to inosine, which is converted to guanosine during reverse transcription by the virus. They are best known for 'editing' mRNAs in the central nervous system to produce different protein isoforms. However, ADARs can also cause large clusters of mutations (hypermutation) in RNA viruses in vertebrates (Carpenter *et al.*, 2009b). Recently, strains of DMelSV have been isolated with patterns of hypermutation characteristic of ADAR. Although this hypermutation could simply be an accident with little effect on viral replication, it is also possible that ADAR may be acting as an

antiviral defence, by disrupting viral gene function or tagging viruses for degradation (Carpenter *et al.*, 2009b).

Coevolution and population dynamics

Drosophila melanogaster

Hosts and pathogens are engaged in a battle of adaptation and counter-adaptation, in which the spread of alleles that increase host resistance is followed by the spread of pathogen genotypes that can overcome these host defences (Dawkins and Krebs, 1979). The *ref(2)P* gene in *D. melanogaster* and the sigma virus is one of the best understood cases of this process.

The resistant allele of *ref(2)P* has arisen from an ancestral susceptible allele, and analyses of DNA sequence variation show that natural selection has caused it to recently increase in frequency in the population (Wayne *et al.*, 1996; Bangham *et al.*, 2007). Based on the degree of linkage disequilibrium with flanking markers, the resistant allele appears to have spread through the host population in the last few thousand years (Bangham *et al.*, 2007). The resistant allele is currently at frequencies of about 24% in natural populations (Wilfert and Jiggins, 2010a), so about 5% of flies will be homozygous and fully resistant (heterozygous flies only transmit the virus at rates slightly less than homozygous susceptible flies; Bangham *et al.*, 2007).

The spread of the resistant *ref(2)P* allele has led to reciprocal evolution in the viral population. In natural populations there are two viral types, one of which can infect the *ref(2)P* resistant flies ('insensitive' viruses) and one of which cannot infect resistant flies ('sensitive' viruses). These insensitive viral types have arisen relatively recently, as European isolates of DMelSV have been estimated to have shared a common ancestor about 200 years ago (Carpenter *et al.*, 2007). This suggests that the mutation that has allowed the virus to overcome host resistance is very recent, as current DMelSV isolates include both the insensitive and a small proportion of sensitive strains (about 16–27%; L. Wilfert, personal communication). The insensitive viral type then rapidly swept through natural host populations in France and Germany over a period of about ten years in the 1980s, largely replacing the original sensitive viral genotypes (Fig. 8.4) (Fleuriet and Sperlich, 1992; Fleuriet and Periquet, 1993). Therefore, this interaction appears to be a classic evolutionary arms race (Dawkins and Krebs, 1979), in which a newly evolved form of host defence rapidly failed owing to evolution in the viral population.

Fleuriet (1982) recreated this process in experimental populations of *D. melanogaster*. Initially the population contained only the susceptible *ref(2)P* allele and was infected with the

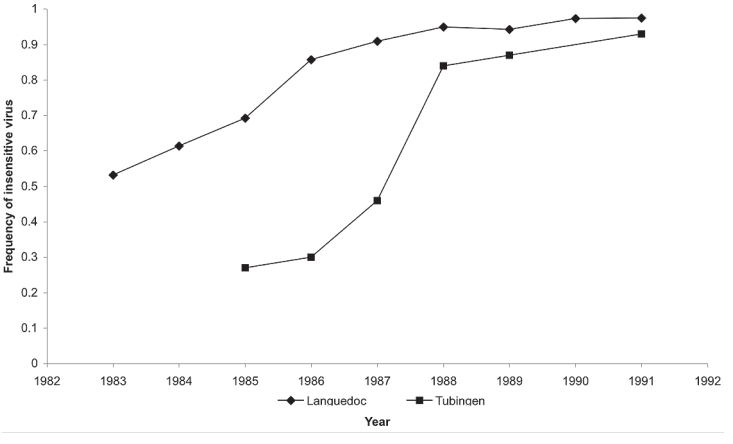

Figure 8.4 Relative frequency of the insensitive viral type (which can infect *ref(2)P* resistant flies) over time in two natural European populations of DMelSV. The figure was drawn using data from Fleuriet and Sperlich (1992) and Fleuriet and Periquet (1993).

sensitive strain of DMelSV. When resistant *ref(2)P* alleles were introduced into this population, their frequency increased to levels similar to that observed in natural populations (about 30%) and there was a decrease in the prevalence of the virus (Fleuriet, 1982). The insensitive viral strain was introduced into the population and replicating the observations in the field, replaced the original sensitive viral genotype, with the prevalence of this virus increasing (Fleuriet, 1982). This also resulted in the resistant *ref(2)P* allele decreasing in frequency, which could be due to a fitness cost of carrying this resistance allele, conveying a selective advantage to the susceptible allele in the absence of a sensitive virus. However, this result must be treated with caution. These experiments were founded with only a few flies and *ref(2)P* is in a region of the genome with low recombination, so there may have been linkage-disequilibrium between *ref(2)P* and deleterious mutations in other genes.

It has also been reported that there is a fitness cost for the virus to be infective and overcome host resistance (Fleuriet, 1999). Fleuriet (1999) attempted to examine this by measuring the frequency of competing insensitive and sensitive viral types in populations of flies lacking the *ref(2)P* resistance allele. The sensitive viral types that were affected by *ref(2)P* resistance had a slight advantage over the insensitive viral types that had evolved to evade this resistance, with about a 20% reduction in male transmission of the insensitive strain (Fleuriet, 1999). However, as this study used only one or two isolates of each of the viral types, the effect may also be due to other deleterious mutations in the viral genome, which has shown no sign of recombination (Carpenter *et al.*, 2007; L. Wilfert, unpublished data).

Drosophila obscura

In populations of *D. obscura* in the United Kingdom (UK), DObsSV shows evidence of even more rapid dynamics than have been observed for DMelSV (Longdon *et al.*, 2011b). In samples from across the UK, the DObsSV viral genomes have extremely low genetic diversity and most single nucleotide polymorphisms (SNPs) occur at a low frequency, suggesting that this virus has very recently spread though the population (Longdon *et al.*, 2011b). Using an approach based on coalescent theory to reconstruct the past population dynamics of the virus, it was estimated that in approximately the past 11 years a single viral clone has spread across the UK to infect about 40% of flies, during which time the viral population size doubled approximately every 9 months (Longdon *et al.*, 2011b). This may have resulted from the spread of the virus through a previously uninfected host population, or a selective sweep of the virus carrying an advantageous mutation through a pre-existing viral population.

Such rapid changes in prevalence are common in pathogens that spread infectiously, but are more surprising in a virus whose only mode of transmission is vertical. However, by combining a mathematical model with laboratory estimates of the vertical transmission rates of DObsSV, it was possible to show that the unusual biparental transmission of the virus through both eggs and sperm can cause such rapid increases in prevalence. Furthermore, this can occur even if the infection significantly reduces the reproductive success of infected flies (Longdon *et al.*, 2011b).

Concluding statement

To study the co-evolutionary and ecological interactions between host and parasites, studying naturally occurring host–parasite combinations is essential (Little, 2002). As they naturally infect *Drosophila,* sigma viruses have great potential as a model to study the fundamental questions relating to the genetics and evolution of viral diseases, and offer a unique opportunity to study insect–rhabdovirus interactions.

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