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Deformed Wing Virus of Honey Bees – Transmission, Diversity and Impact on Honey Bee Development

by
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Declaration

This thesis is submitted to the University of Warwick for the degree of PhD. I declare that all of the work presented in this thesis was original work performed by myself, except where specifically stated. None of this work has been previously submitted for any other degree.

Summary

The spread of the parasitic mite, *Varroa destructor* (and the RNA viruses that it transmits between honeybees) is closely linked to increased overwintering losses and declining honeybee health. Deformed wing virus (DWV; *Iflaviridae*) a picorna-like single-stranded, positive-sense, RNA virus, is widespread in honeybees and usually present as a low level, asymptomatic infection. However, the transmission of DWV by *Varroa* to developing pupae causes markedly elevated virus levels and characteristic developmental abnormalities.

This work aims to further the understanding of DWV virulence and pathogenicity and reveal how this is linked to *Varroa* parasitism. Molecular techniques and a novel *in vitro* larval rearing system have been used to study how transmission route influences DWV accumulation and localisation in honeybee tissues. Additionally, the impact of *Varroa* on DWV levels and diversity on both a colony and a landscape level have been investigated, testing the hypothesis that a single virulent strain has a selective advantage when transmitted by *Varroa*.

The route of transmission does influence DWV infection and tissue location in developing honeybees, as shown by RNA-FISH. Injection of a dose of 10^{-4} ng of viral RNA was sufficient to produce a pronounced DWV infection in the majority of individuals, whereas 300ng/larvae of DWV RNA needed to be mixed with larval food before any high viral load individuals were found. I also present novel work in which the relative expression levels of key developmental genes from the Homeobox and Ecdysone families are examined in honeybee larvae challenged by injection with DWV. In addition to this, a long-term study of viral diversity in the Ardnamurchan Peninsula, Scotland, illustrates how the viral landscape is affected by the introduction of the *Varroa* mite. *Varroa* colonisation led to a shift from a mixed population of viruses, predominantly VDV-like or VDV/DWV recombinants, towards a mainly DWV-like virus population.

Chapter 1. Introduction

1.1 General Introduction

Insect pollination of agricultural crops and wild plants provides a key ecosystem service, with up to 35% of global crop production (Klein, *et al.*, 2007) and over 85% of wild flowering plants (Ollerton, *et al.*, 2011) utilizing insect pollination services. Consequently, global threats to pollinators (Potts, *et al.*, 2010a) are of great concern to the scientific and agricultural communities and may pose a threat to human and environmental health.

The European honey bee (*Apis mellifera* L.) is the world's most economically important managed pollinator. Honey bees are partially domesticated, and have been used by people for honey production and pollination services for at least 7000 years (Bloch, *et al.*, 2010; Oldroyd, 2012). This association means that many members of the public are aware of, and concerned by, threats faced by honey bees. Globally, stocks of honey bees have increased by approximately 45% during the last half century, predominantly as a result of a rising demand for insect pollinated crops in Asia and South America (Aizen and Harder, 2009). However, there is evidence indicating that we should be concerned about the continuing, long term viability of honey bee stocks, particularly with respect to increasing threats to honey bee health from parasites, pathogens and other natural enemies (Potts *et al.* 2010b). In contrast to the rise in honey bee colony numbers at the global level, the numbers of managed honey bee colonies in western Europe fell by 25% between 1985 and 2005 (Potts, *et al.*, 2010b) and in North America it decreased by 59% from 1959 to 2005 (Mazer, 2007). These declines are associated with documented increases in overwinter colony losses linked to infectious diseases, and which have also occurred in, Asia, Africa and South America (BBKA; vanEngelsdorp, *et al.*, 2009; Goulson, *et al.*, 2015; Chantawannakul, *et al.*, 2016; Maggi, *et al.*, 2016). Rising overwinter losses can be offset to some extent by beekeepers splitting their colonies to make increase in the summer but this is unlikely to be sustainable in the long term, and already the rate of increase of honey

bee stocks globally is not keeping pace with rising demand (Aizen & Harden, 2009). Regional declines in honeybee stocks, and failure to meet increasing demand for their services, could have wide ranging impacts, both for food production and the natural environment (Klein, *et al.*, 2007; Potts, *et al.*, 2010b).

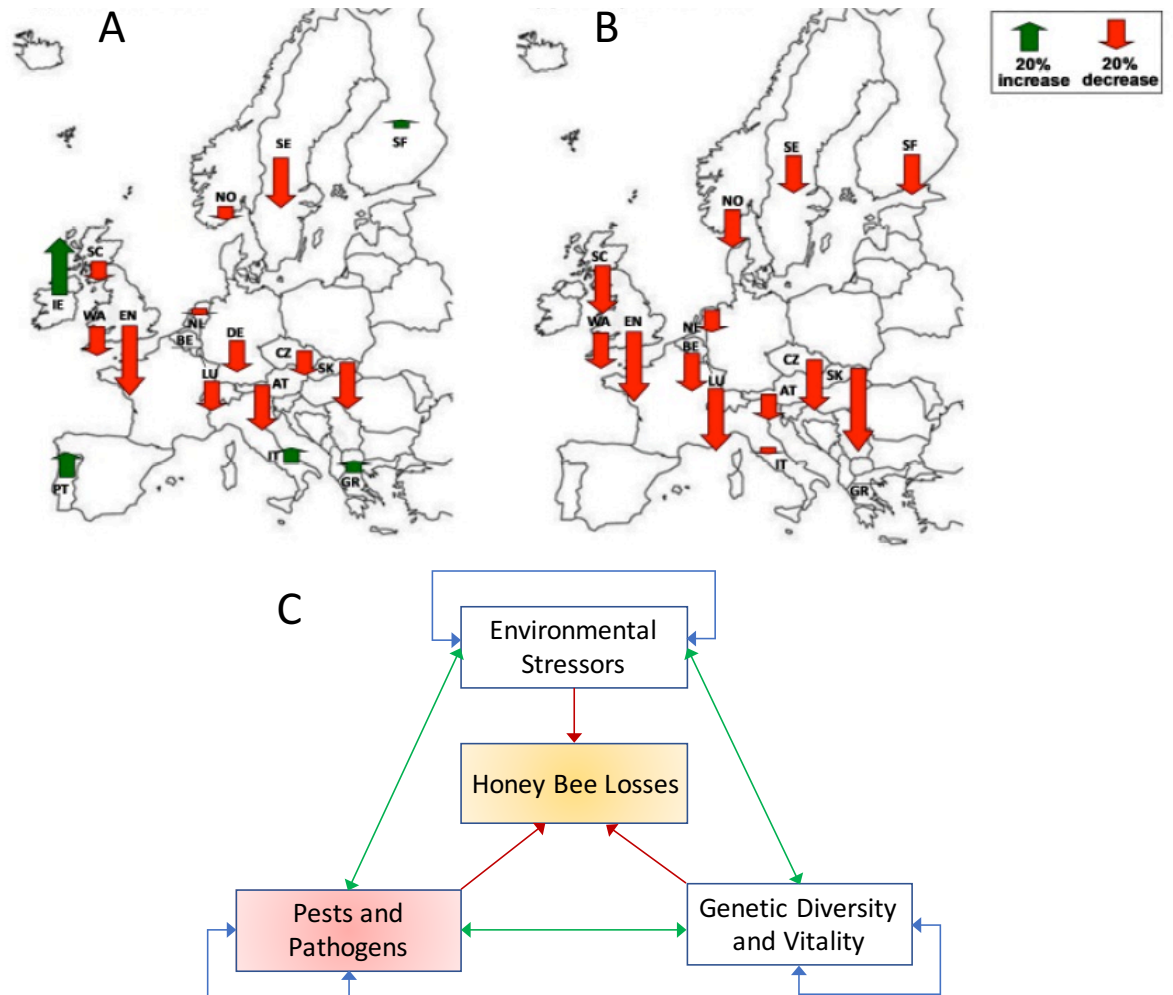


Figure 1.1. European declines in managed honey bee colonies (1985-2005) (A) and beekeepers (1985-2005) (B). Reproduced from (Potts, *et al.*, 2010b). (C) The three main drivers of honey bee loss, with red arrows representing direct pressures from these drivers, green arrows representing interactions between drivers and blue arrows representing interactions within the drivers, figure adapted from (Potts, *et al.*, 2010a). The focus of this work is the impact of pests and pathogens on honey bee health, but it is important to consider how these factors interact with the other main drivers of honey bee losses.

While there are a range of drivers affecting honey bee health (Potts *et al.*, 2010a,b), the spread of the ectoparasitic mite *Varroa destructor* (and the RNA viruses that it carries and transmits between honey bees) is closely linked to the increase in overwintering losses and decline in honeybee health and appears to be a particularly important threat (Anderson and Trueman, 2000; Martin, 2001; Le Conte, *et al.*, 2010). Deformed Wing Virus (DWV) is the pathogen that has been shown to have the closest association with *Varroa* parasitism and honey bee colony losses, and knowledge of how this virus has capitalised on the global spread of the *Varroa* mite will be crucial if we are to develop effective mitigations and conserve honey bees into the future (Allen and Ball, 1996; Bowen-Walker, *et al.*, 1999; Nordstrom, *et al.*, 1999; Sumpter and Martin, 2004; Ribière, *et al.*, 2008; de Miranda and Genersch, 2010)

1.1 The Biology of the Honey Bee

Honey bees (Hymenoptera, Apidae, subfamily Apinae, tribe Apini) are closely related to the orchid bees (tribe Euglossini), the bumble bees (tribe Bombini) and the stingless bees (tribe Meliponinae). These tribes are characterized by the presence of a pollen basket on the hind tibia and the exhibition of some degree of social behaviour (Winston, 1987). There are five extant species of honey bees: the European (aka the common or western) honey bee *Apis mellifera*, the Asian honey bee *Apis cerana*, the giant honey bees *Apis dorsata* and *Apis laboriosa*, and the dwarf honey bee *Apis florea*. The natural habitat of the western honey bee reaches from northern Europe down to the southern tip of Africa, and its range has been further extended into Asia, The Americas and Australasia through domestication and beekeeping (Winston, 1987).

Apis mellifera is a eusocial insect, characterised by division of labour according to sex and age, and cooperative rearing of offspring. A typical *A. mellifera* colony is made up of a multiple comb nest within a cavity (be that naturally occurring or man-made) and contains a single reproductive female (known as the queen), 20,000 to 60,000 non-

reproductive females (or workers), 10,000 to 30,000 juvenile non-reproductive females (worker brood) and several hundred reproductive males (drones). Typically, honey bee workers develop to adulthood in 21 days, with queens taking 16 days and drones taking 24 days (Winston, 1987) (Fig 1.2 A).

As eusocial insects, honey bees have developed a complex system of social organisation, defined by reproductive division of labour (with reproduction being the domain of the queen and drones, and all other tasks being carried out by female workers), the cooperative care of offspring and the presence of overlapping generations within the colony (Wislon, 1971; Schmid-Hempel, 1998). Living in these large, complex societies improves honey bees' ability to compete for resources against smaller groups or solitary individuals but is also associated with the rapid spread of pathogens due to close physical contact within crowded colonies (Schmid-Hempel, 1998; Hughes, *et al.*, 2002; Holldobler and Wilson, 2008). This means that honey bees invest heavily in pathogen defences, including inducible cellular and humoral immunity, the acquisition and secretion of antimicrobial compounds and group defences such as hygienic behaviour (Arathi, *et al.*, 2000; Castella, *et al.*, 2008; Fernandez-Marin, *et al.*, 2009; Wilson-Rich, *et al.*, 2009). The genetic diversity within a colony, created by multiple queen matings (polyandry) also plays a role in disease prevention (Boomsma; Tarpy, 2003; Seeley and Tarpy, 2007).

Within honey bee colonies, as with all eusocial insect societies, the majority of tasks within the colony are performed by the functionally sterile adult female workers (Kolmes, 1986). Workers are involved in the tasks of comb building and cleaning, tending to the developing brood, feeding and responding to the queen, colony ventilation, guarding the colony and foraging for pollen and nectar. Worker task allocation is temporal, with the risky duties such as foraging being carried out by older individuals (Tofilski, 2002). During the spring and summer, newly emerged bees or 'house' bees perform housekeeping duties inside the colony such as nectar and pollen processing and brood care, and then transition to foraging duties at around 23 days post emergence (Winston, 1987). In contrast, worker bees produced in the autumn are needed to ensure the survival of the colony during the winter whilst no brood is

produced and no foraging occurs, they remain inside the colony and can live for up to 6 months (Winston, 1987). The queen is responsible for egg laying, and only leaves the colony for mating flights or during swarming. Drones are present during the early part of the season and their sole function is to mate with queens (Winston, 1987).

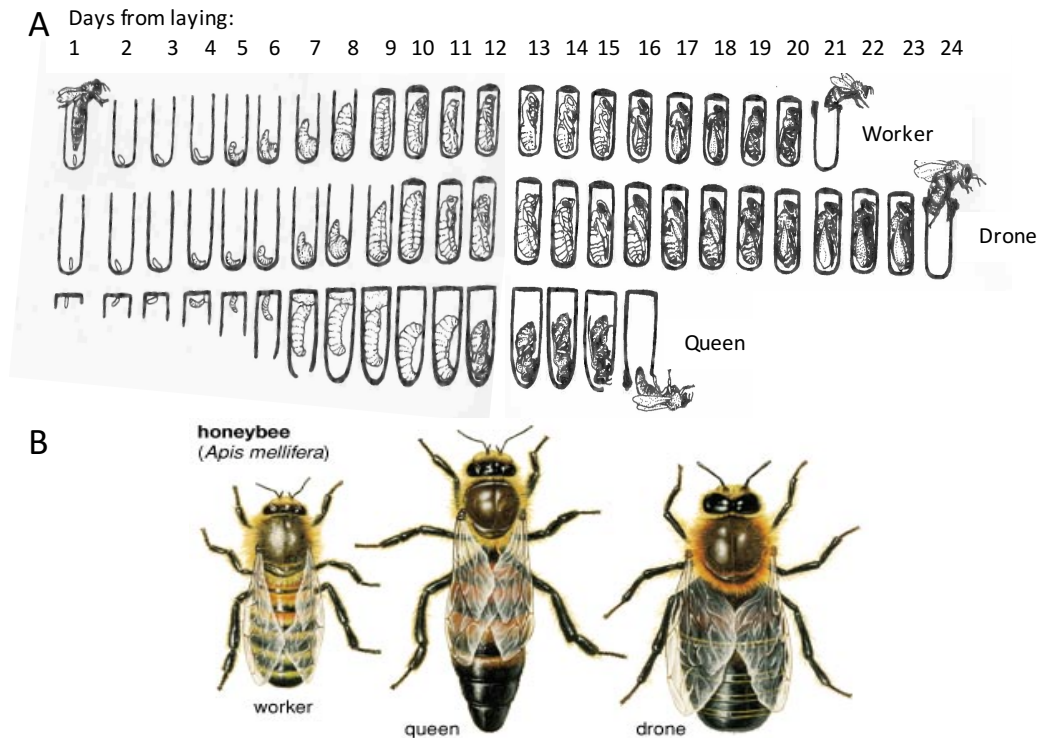


Figure 1.2 (A) Developmental times for workers, drones and queens, figure adapted from Winston (1987). (B) The honey bee worker, queen and drone. The queen and worker are female and diploid. The drone is male and haploid. Picture credit, Encyclopaedia Britannica ImageQuest Database.

1.2 *Varroa destructor* – History, Taxonomy, Global Distribution and Biology

Varroa mites (Acari: Varroidae) are highly specialized, obligate parasites of eusocial bees, first described by Oudemans in Java, Indonesia in 1904, feeding on the haemolymph of *A. cerana* (Anderson and Trueman, 2000). *Varroa* mites were able to expand their host range from their natural host, *A. cerana*, to *A. mellifera*, when the latter began to be kept by beekeepers in areas where *A. cerana* is endemic (Oldroyd, 1999a). *Apis mellifera* proved to be a far more susceptible host due to differences

between the species in brood development and, with the assistance of modern, globalized beekeeping practices, *Varroa* has been spread across most of the world. It is thought that this host species jump marked the speciation of the originally described mite (known as *Varroa jacobsoni*) into two species – which are differentiated by genetic markers and by the ability to reproduce in *A. mellifera* colonies (Anderson and Trueman, 2000; Rosenkranz, *et al.*, 2010). The new species, able to successfully parasitize *A. mellifera*, was described by Anderson and Trueman in 2000 and is known as *Varroa destructor*. In this text, the term *Varroa* refers to *Varroa destructor*.

From its origins in Asia, *V. destructor* has been spread across the majority of the globe as a result of the international trade in honey bees. It is present on every continent where beekeeping takes place, with Australia being the only remaining large land mass that is *Varroa* free (Beverley, 2017). *Varroa* mites were reported in *A. mellifera* colonies in Eastern Russia in 1952, China in 1959, Paraguay in 1971, Germany in 1977 and the USA in 1987 (Rosenkranz, *et al.*, 2010). They were first recorded in the UK in 1992 and, by 2005, were considered to be ubiquitous across England and Wales, and widespread across Northern Ireland and Scotland (NationalBeeUnit, 2010).

Infestations by *Varroa* are considered to be one of the key causes of honey bee colony loss, particularly through overwintering losses (Potts *et al.*, 2010b). Details of the biology of *Varroa*, and of its role as a vector of bee pathogenic viruses, particularly deformed wing virus, are described in the following sections of this chapter.



Figure 1.3. Map of known *Varroa destructor* distribution, as published by the Invasive Species Compendium, www.cabi.org/isc.

Varroa mites are obligate ectoparasites of honey bees and their life cycle takes place within the hive. The mites demonstrate distinct sexual dimorphism and are haplodiploid, with the adult female having a reddish-brown, oval shaped body 1.1 to 1.2mm in length and 1.5 to 1.6mm in width. Male mites are smaller (approximately 0.7mm in width and length) and very pale tan in colour (Rosenkranz, *et al.*, 2010).

The female mite has two life phases – the phoretic phase, where it is attached to an adult bee and able to travel in this way between honey bee colonies, and the reproductive phase which takes place inside sealed drone or worker brood cells. The reproduction cycle begins with a female entering a cell immediately prior to capping. She then lays one unfertilised egg (which hatches into a male) and then several fertilised eggs which develop into females. Mating between the hatched offspring occurs within the sealed cell, allowing several fertilised females to emerge along with the bee once it reaches adulthood. Males and any immature female nymphs remain in the cell and die after the bee emerges (Oldroyd, 1999b; Rosenkranz, *et al.*, 2010). Details of the timing of these events are given in Fig 1.4. Mites exhibit a preference for reproduction within honey bee drone brood, with an 8 to 10-fold higher infestation rate in male brood (Boot, *et al.*,

1995; Rosenkranz, *et al.*, 2010) and, because the capped stage of drone brood extends for two days further than that of worker brood, more mites go on to reach maturity. In drone cells two to three viable female offspring are produced for each foundress mite, whereas in worker cells one or two female offspring reach maturity (Schulz, 1984). However, drone production is seasonal, peaking in spring and early summer, and once this period is over the *Varroa* load in worker brood increases (Rosenkranz and Renz, 2003)

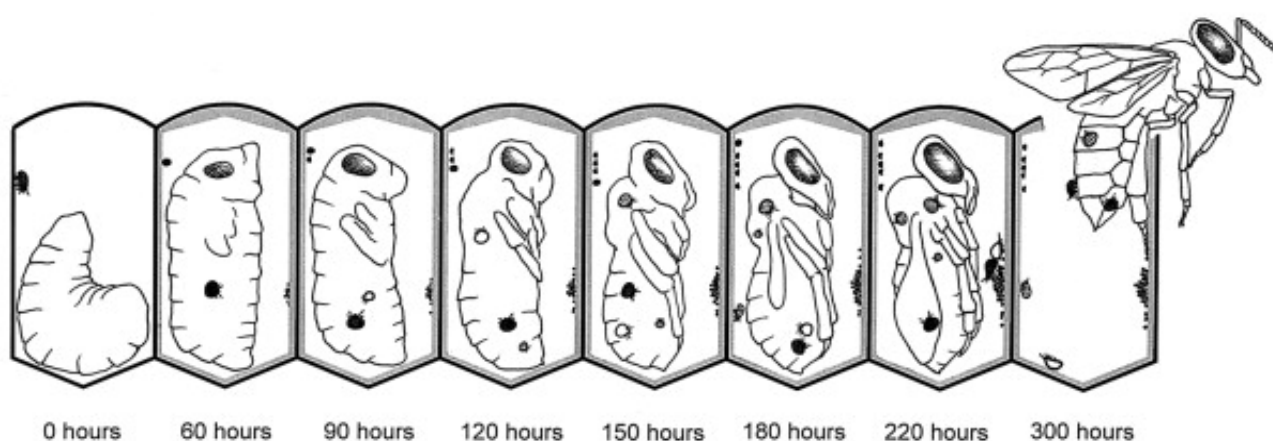


Figure 1.4. The reproductive life cycle of *V. destructor* on *A. mellifera* worker brood. At 0h the mature female enters the cell. At 60h the male egg has been laid close to the top of the cell and the mother has opened a feeding hole on the developing pupae. By 90h the male nymph has hatched and the first female egg has been laid. A new daughter egg is laid every 30 hours. Once the male and female offspring reach adulthood (starting at 220 hours post capping) they mate on the side of the cell. At 300 hours the newly adult honey bee emerges, along with the mother mite and any mature, mated daughters. The male and any immature females stay in the cell to die. Figure from (Oldroyd, 1999b).

All life stages of the *Varroa* mite feed on honey bee haemolymph, either from the developing pupae in the reproductive phase, or from the adult bee during the phoretic stage. It is through this feeding that the honey bee is damaged by *Varroa*. The loss of haemolymph from the developing pupae is energetically costly, reducing the weight of the emerging worker bee by (on average) 7%, and by as much as 19% in drones (Dejong, *et al.*, 1982; Duay, *et al.*, 2003). Worker bees which were exposed to *Varroa* feeding during their development have an altered physiology, life pattern (starting their

foraging stage earlier) and a significantly shorter life span (Dejong, *et al.*, 1982; Amdam, *et al.*, 2004). Foraging behaviour and ability is also affected, with parasitized individuals exhibiting longer absences from the colony and a reduced return rate from foraging (Kralj and Fuchs, 2006; Kralj, *et al.*, 2007).

1.3 *Varroa* as a Vector of Honey Bee Pathogenic Viruses

There is general consensus among honeybee researchers that *Varroa destructor*'s close association with honey bee viruses plays a significant part in explaining why their introduction to *A. mellifera* has proved so costly in terms of honeybee health (Sumpter and Martin, 2004; Shen, *et al.*, 2005a; Shen, *et al.*, 2005b; Genersch and Aubert, 2010; McMenamin and Genersch, 2015). *Varroa* mites can transmit multiple viruses to their hosts, including deformed wing virus (DWV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and Israeli acute paralysis virus (IAPV) and these viruses, not the mites themselves, may be responsible for a significant proportion of the harm that occurs in bees when they are parasitized by *Varroa* (Bowen-Walker, *et al.*, 1999; Cox-Foster, *et al.*, 2007; Carreck, *et al.*, 2010). Viruses transmitted by feeding mites may be able to pass directly into the developing pupae or adult bee's haemolymph, thus bypassing the usual routes of transmission and therefore the honeybees' existing defences (Shen, *et al.*, 2005a).

The introduction of *Varroa* to an area has been shown to have a dramatic impact on the viral landscape within honey bee colonies. Prior to the introduction of *Varroa* mites, honey bee viruses were known to be present in the UK, but were often at very low titres – below the detection thresholds of the most commonly used techniques of the time (Bailey, *et al.*, 1981). Sampling of bees from recently *Varroa* infested UK colonies in Devon and Herefordshire between 1993 and 1997, revealed that three honey bee viruses were repeatedly found at higher levels in *Varroa* exposed bees compared to their recorded occurrence (both in terms of virus titre per bee and virus prevalence within a colony) prior to *Varroa* introduction (Carreck, *et al.*, 2010). These viruses were DWV, cloudy wing virus (CWV) and slow paralysis virus (SPV). The presence of one or more

of these viruses was also strongly associated with over winter colony loss. There have been a number of wide-ranging honey bee disease monitoring projects, prompted by the international concern over the dramatic increase in honey bee colony losses over the last 20 years. The National Bee Unit (FERA, UK) have published the results of their comprehensive sampling and monitoring project, which gives an excellent picture of the viral prevalence in colonies in England and Wales after the introduction of the *Varroa* mite (sampling took place between 2007 and 2008). As anticipated, weaker colonies were found to be more frequently associated with higher numbers of pathogens. One key finding of the survey was that, of all of the pathogens tested for, DWV was the only one that could be reliably used as an indicator of colony strength, with colonies that had high levels of DWV being consistently more likely to have fewer combs of brood or bees (Budge, *et al.*, 2015). A similar project in Germany found that high levels of DWV and acute bee paralysis virus (ABPV) in the autumn, along with a heavy *Varroa* mite load, were significantly related to overwinter colony losses (Genersch, *et al.*, 2010). DWV has also been implicated in the rise in colony losses in Greece (Bacandritsos, *et al.*, 2010) and Switzerland (Dainat, *et al.*, 2012b). However the picture is less clear in the USA, where research has focused on ‘Colony Collapse Disorder’ (CCD), with DWV being amongst the set of pathogens that were found to be moderately higher in colonies that had suffered from CCD in one study (Cornman, *et al.*, 2012), but not amongst the most significant predictors reported by van Engelsdorp, *et al.*, (2009).

The link between *Varroa* transmitted honey bee viruses and colony loss is intensified by the dynamics of brood rearing and *Varroa* load within the colony. The workers reared in late summer and autumn are the overwinter bees, and they need to live for up to 6 months during the winter period whilst no brood is reared (Winston, 1987). During the time when these overwinter bees are being reared, drone brood production ceases and mite population continues to increase, leading to a substantial rise in the proportion of workers infested with *Varroa* mites (Martin, 1998; Rosenkranz and Renz, 2003). A modelling approach has shown that this leads to reduced lifespan in these individuals caused by DWV infection, which impacts the age structure of the overwintering bee population and results in colony death as insufficient bees remain to sustain the colony (Martin, 2001)

1.5 Deformed Wing Virus – Classification, Genetics and Structure

Deformed wing virus (DWV; *Iflaviridae*) a picorna-like single-stranded, positive-sense, RNA virus, is one of many viruses infecting honeybees and one of the most heavily investigated due to its close association with *Varroa* mites and honey bee overwintering losses.

The genus Iflavirus includes diverse RNA viruses of arthropods, which have icosahedral non-enveloped virus particles about 26 to 32 nm in diameter (Figure 1.5 A) (Chen, *et al.*, 2012). Iflavirus virions contain a single copy of linear positive-sense 8 to 10 kb genomic RNA which possesses small genome-linked virus protein, Vpg, covalently linked to the 5' terminus and poly(A) sequence at the 3' terminus. Genomic RNAs of iflaviruses have extended 5' untranslated region (5'UTR), reaching about 1 kb, which acts as an internal ribosome entry signal, IRES. The IRES is followed by a single open reading frame (ORF) coding for a large polyprotein, ranging in size from 2800 to 3200 amino acids, with the structural proteins and the non-structural proteins encoded by the 5'- and 3'-parts of the ORF, respectively. Iflavirus genomic RNA is infectious and serves as a template for the synthesis of viral polyprotein. The genus name is derived from the name of its type species, **I**nfectious **f**lacherie virus, a pathogen of the silkworm, *Bombyx mori* (Isawa, *et al.*, 1998).

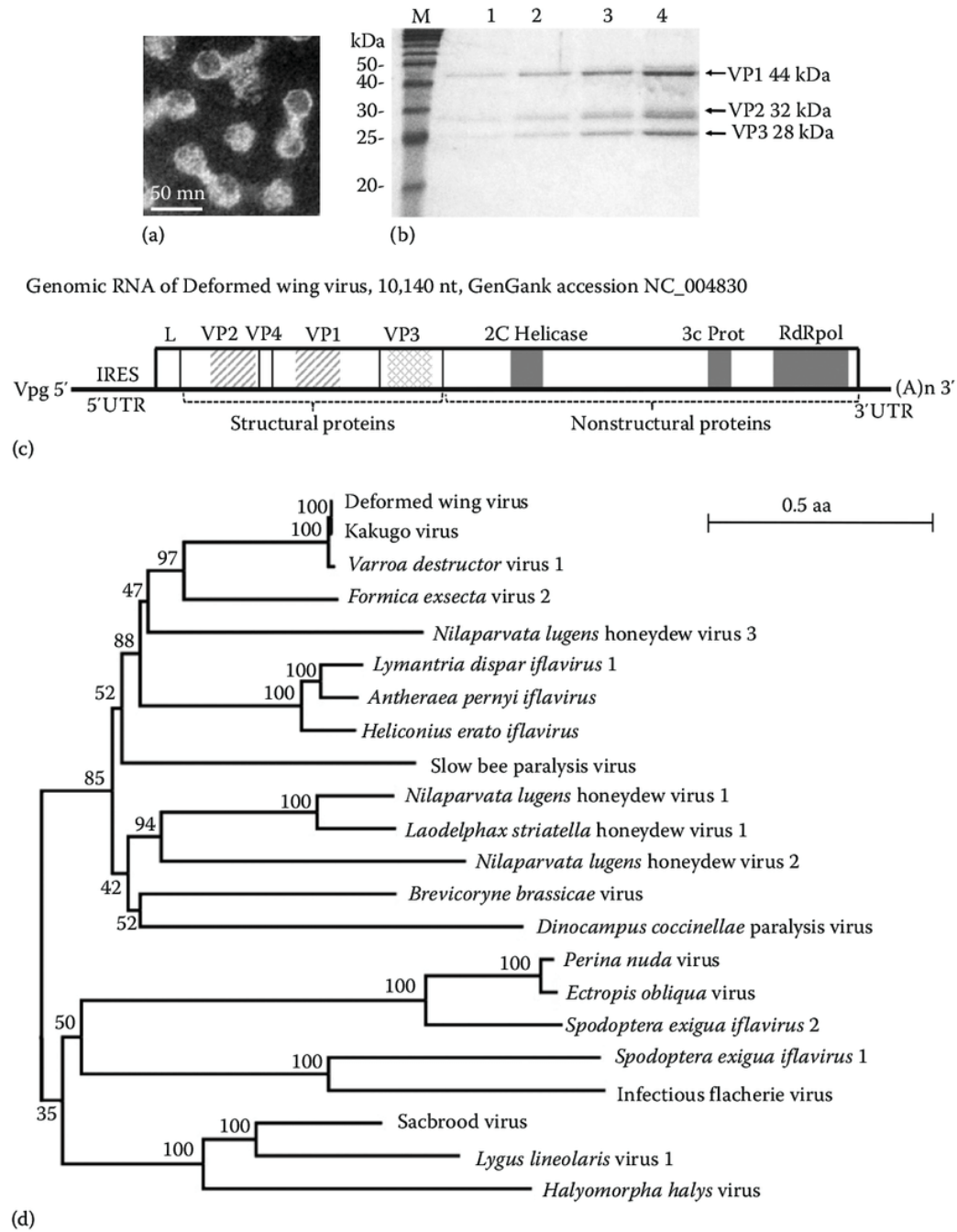


Figure 1.5 (a) Transmission electron photograph of deformed wing virus (DWV) particles, bar 50 nm. (b) SDS PAGE of DWV virions showing three major structural proteins, VP1, VP2, and VP3. Lane M, molecular weight protein markers; lanes 1–4, dilutions of virus preparation. (c) Organization of DWV genomic RNA. The long box represents the single open reading frame with the conserved domains indicated. Vertical lines represent identified cleavage sites. Conserved domains are indicated as follows: boxes in the VP2 and VP1 proteins shown are picorna-like capsid drug-binding pocket domains, box in

VP3 is a CrPV capsid-protein-like domain, and dark boxes in the nonstructural part are picorna-like 2C helicase, 3C protease, and RNA-dependent RNA polymerase (RdRp) domains. (d) Phylogram of the recognized and putative members of the genus Iflavirus. Full-length polyprotein sequences of the iflavirus isolates listed in Table 1.1 were used. The tree was produced using the neighbour-joining method and evaluated with bootstrap analysis, 1000 replicates, percentage of bootstrap support if each branch is indicated. Branch length indicates evolutionary distance; scale bar shows 0.5 amino acid substitutions per site. Figure adapted from (Fannon and Ryabov, 2016)

In recent years, more viruses with Iflavirus characteristics have been discovered in a variety of arthropod species mostly as a result of transcriptome sequencing (see table 1.1 for details and references). Despite high variation between the polyproteins of different iflaviruses, they share a number of common features. The short N-terminal Leader protein (L) of unknown function, which is highly diverse between iflaviruses, precedes more conserved block of the structural proteins with three domains typical for the capsid proteins of icosahedral picornaviruses (VP1, VP2, VP3) (shown in Figure 1.5 B), and the smallest structural protein VP4, which are arranged in the order VP2-VP4-VP1-VP3 in the polyprotein (Lanzi, *et al.*, 2006). The structural proteins are proteolytically processed by the 3C protease domain encoded by the non-structural block, and precise processing sites have been experimentally determined for several iflaviruses, including Deformed wing virus (Lanzi, *et al.*, 2006) and *Lymantria dispar* iflavirus 1 (Carrillo-Tripp, *et al.*, 2014) (Figure 1.5 C). The C-terminal half of the iflaviral polyprotein contains domains with the motifs typical for picornaviral 2C RNA helicase, a chymotrypsin-like 3C protease and 3D RNA-dependent RNA polymerase (Figure 1.5 C) (Koonin and Dolja, 1993). No experimental data is available on the processing of the non-structural portion of iflavirus polyprotein, although it is suggested that processing of the non-structural proteins is facilitated by the 2C protease similar to that of the picornavirus polyproteins.

The genus Iflavirus covers a group of positive-sense ssRNA viruses in the family Iflaviridae, order Picornavirales. Currently, only six species are recognized in the genus

Iflavirus according to the ICTV report published in 2012 (Chen, *et al.*, 2012) (Table 1.1). At least 17 additional insect RNA viruses with fully sequenced genomes (or at least with fully sequenced protein coding regions) are putative members of the genus according to their genome organization and to their being significantly different from other iflaviruses (nucleotide [nt] identity less than 90%) (Table 1.1). Among the evolutionarily close iflaviruses, similarity can very high; the nt and aa identity between closely related honey bee viruses DWV and Varroa destructor virus 1 (VDV-1) are as high as 84% and 95%, respectively; and lepidopteran *Perina nuda* virus (PnV) and *Ectropis obliqua* virus (EoV) have 82% nt and 87% aa identity.

The phylogenetic tree shown in figure 1.5 (d) shows how DWV falls into a clade with two other characterised iflaviruses, Kakugo virus (KV) and Varroa destructor virus-1 (VDV-1). KV was first isolated from the brains of aggressive honey bees in Japan (Fujiyuki, *et al.*, 2004) and it is so closely related to DWV that there is some disagreement as to whether the two are actually regional isolates of the same virus (Lanzi, *et al.*, 2006) or two distinct species (Fujiyuki, *et al.*, 2006). However, unlike KV, DWV has never been associated with an increase in the aggressiveness of infected bees (Rortais, *et al.*, 2006). VDV-1 was first isolated from *Varroa*, taken from honey bee colonies in the Netherlands (Ongus, *et al.*, 2004), and once identified, was found to be widely prevalent in honey bees, often co-occurring and recombining with DWV-like viruses (Moore, *et al.*, 2011; Ryabov, *et al.*, 2017).

DWV, KV and VDV-1 exhibit 84% sequence homology, and can be considered as strains of the same virus (Ryabov, *et al.*, 2014a). The nucleotide differences between the three characterized viruses are mostly located at the 5' end of the genome, chiefly in the 5' UTR and the Lp region (Lanzi, *et al.*, 2006; de Miranda and Genersch, 2010). The strains have been shown to recombine (Moore, *et al.*, 2011), and can be seen as a viral quasispecies or variants of the same species complex. The quasispecies hypothesis refers to an equilibrium in the process of mutation and natural selection, leading to a

genetically diverse population (Domingo, *et al.*, 2012). These diverse variants are clustered around one or more ‘master sequence’ genotypes, those of the highest fitness in a particular environment or niche (Domingo, *et al.*, 2012). The small genomes and high replication and mutation rates of RNA viruses contribute to their highly genetically diverse populations, and viral evolution is often explained in terms of quasispecies concepts (Domingo and Holland, 1997; Holmes and Moya, 2002; Stich, *et al.*, 2007).

For this thesis, DWV or DWV-like will be used to refer to all strains of the virus that are similar to the full length Deformed Wing Virus sequence, as is detailed in Lanzi *et al* (2006), RefSeq [NC_004830.2](#) Accession Number AJ489744. VDV-1 or VDV-like refers to viral sequence deemed to be similar to the Varroa Destructor Virus-1 sequence from (Ongus, *et al.*, 2004), RefSeq [NC_006494.1](#), Accession Number AY251269. Similarity is determined by BLAST alignment of sequenced samples and by strain specific PCR and qPCR (see details in Chapter Two).

There has been a trend in recent publications to refer to DWV and KV type viral sequences as DWV type A, and VDV type viral sequences as DWV type B (McMahon, *et al.*, 2016b; Mordecai, *et al.*, 2016a; Mordecai, *et al.*, 2016b). This is an interesting development, as it serves to illustrate how these strains can be seen as members of the same species complex rather than as distinct taxa. However, currently only two research groups have adopted this change in nomenclature, and a consensus has not been reached yet from the research community about an agreed nomenclature. For clarity, the original DWV/VDV-1 definitions will be used in this work in line with the majority of published research.

The three-dimensional structures of DWV have recently been determined by cryo-electron microscopy and X-ray crystallography (Skubnik, *et al.*, 2017). The DWV virion contains a C-terminal extension of the capsid protein VP3, which forms a

globular domain containing a predicted receptor binding site on the virion surface. DWV's active site is predicted to be flexible, adopting two conformations according to pH or ionic conditions, and it is hypothesized that this variable p domain may be involved in catalytic activity for cell entry.

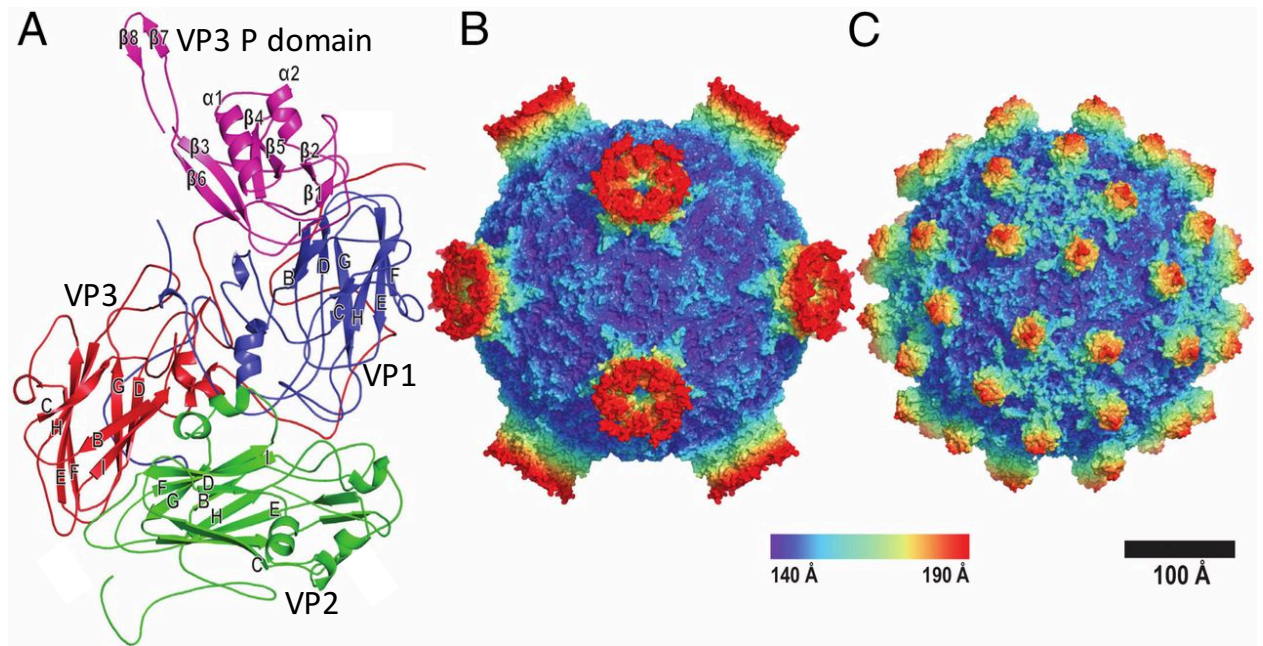


Figure 1.6 Structures of the icosahedral asymmetric unit of DWV and its virions in alternative conformations, figure from (Skubnik, et al., 2017). (A) The icosahedral asymmetric unit of DWV in schematic representation, major capsid protein VP1 shown as blue, VP2 shown as green, and VP3 shown as red. The P domain (part of VP3) is magenta. The molecular surfaces of DWV virions were determined by cryo-electron microscopy (shown in B) and by X-ray crystallography (shown in C). The virion surfaces have been colored to illustrate their distance from the midpoint of the particle. (Scale bar: 100 Å.)

Table 1.1 Members of the Genus *Iflavirus* with Full-Length Genomic RNA Sequences

Isolate Name (Acronym)	GenBank Accession Number	Host Name/Order	Common	References
Members of the genus <i>Iflavirus</i> recognized by the ICTV, 2012 report (Chen, <i>et al.</i>, 2012)				
Infectious flacherie virus (IFV)	NC_003781	Moths/Lepidoptera		(Isawa, <i>et al.</i> , 1998)
Perina nuda virus (PnV)	NC_003113	Moths/Lepidoptera		(Wu, <i>et al.</i> , 2002)
Ectropis obliqua virus (EoV)	NC_005092	Moths/Lepidoptera		(Wang, <i>et al.</i> , 2004)
Deformed wing virus—(DWV)	NC_004830	Honeybee/Hymenoptera Mites/Arachnida		(Lanzi, <i>et al.</i> , 2006)
Deformed wing virus—Kakugo virus (KV)	NC_005876	Honeybee/Hymenoptera		(Fujiiyuki, <i>et al.</i> , 2004)
Varroa destructor virus 1— (VDV-1)	NC_006494	Honeybee/Hymenoptera Mites/Arachnida		(Ongus, <i>et al.</i> , 2004)
Sacbrood virus— (SBV)	NC_002066	Honeybee/Hymenoptera		(Ghosh, <i>et al.</i> , 1999)
Putative members of the genus <i>Iflavirus</i>				
Formica exsecta virus 2 (Fex2)	KF500002	Ants/Hymenoptera		(Johansson, <i>et al.</i> , 2015)
Nilaparvata lugens honeydew virus 1 (NLHV-1)	AB766259	Planthopper/Hemiptera		(Murakami, <i>et al.</i> , 2013)
Nilaparvata lugens honeydew virus 2 (NLHV-2)	NC_021566	Planthopper/Hemiptera		(Murakami, <i>et al.</i> , 2013)
Nilaparvata lugens honeydew virus 3 (NLHV-3)	NC_021567	Planthopper/Hemiptera		(Murakami, <i>et al.</i> , 2013)
Lymantria dispar iflavirus 1 (LdIV1)	KJ629170	Moths/Lepidoptera		(Carrillo-Tripp, <i>et al.</i> , 2014)
Antheraea pernyi iflavirus—LnApIV (ApIV)	KF751885	Moths/Lepidoptera		(Geng, <i>et al.</i> , 2014)
Heliconius erato iflavirus (HelIV)	KJ679438	Butterflies/Lepidoptera		(Smith, <i>et al.</i> , 2014)
Slow bee paralysis virus (SBPV)	NC_014137	Honeybees/Hymenoptera		(de Miranda, <i>et al.</i> , 2010)
Laodelphax striatella honeydew virus 1 (LSHV1)	KF934491	Planthopper/Hemiptera		
Brevicoryne brassicae virus (BrBV)	NC_009530	Aphids/Hemiptera		(Ryabov, 2007)
Dinocampus coccinellae paralysis virus—	KF843822	Parasitoid wasp/Hymenoptera		(Quebec, 2013)
Spodoptera exigua iflavirus 1 (SeIV-1)	NC_016405	Moths/Lepidoptera		(Millan-Leiva, <i>et al.</i> , 2012)
Spodoptera exigua iflavirus 2 (SeIV-2)	JN870848	Moths/Lepidoptera		(Choi, <i>et al.</i> , 2012)

Lygus lineolaris virus 1 (LyLV-1)	JF720348	Plant bug/Hemiptera	(Perera, <i>et al.</i> , 2012)
Halyomorpha halys virus—Beltsville	NC_022611	Stink bug/Hemiptera	(Sparks, <i>et al.</i> , 2013)

1.6 Deformed Wing Virus – history, spread and global prevalence

DWV was first named and characterised in Japan in 1982, when a virus was isolated from symptomatic honey bees in a colony infested with *Varroa*. The bees had stunted abdomens and deformed wings, hence the name given to the virus (Ribière, *et al.*, 2008). Wing deformities and reduced abdominal size began to be widely reported in honey bee colonies following the spread of *Varroa destructor*, and these symptoms were initially attributed to the feeding activities of the mite. However, it became evident that not all *Varroa* exposed bees developed these symptoms, and that some individuals in heavily infested colonies had the deformities despite having no direct *Varroa* exposure (Koch and Ritter, 1991; Marcangeli, *et al.*, 1992). Further research revealed that experimental injection of DWV causes the characteristic symptoms in *Varroa* naïve bees (Moeckel, *et al.*, 2011). DWV is now known to be present in honey bee colonies globally, having been detected in bee samples from Europe, Africa and Asia (Allen and Ball, 1996) and in North, South and Central America (Ellis and Munn, 2005; Maggi, *et al.*, 2016). The picture is more complicated in Australasia, with DWV being present in New Zealand where *Varroa* has recently been introduced (Mondet, *et al.*, 2014; Iwasaki, *et al.*, 2015) but not in Australia which remains *Varroa* free (Roberts, *et al.*, 2017).

In the UK, a virus later shown to be DWV was first identified in the early 1980's, before the introduction of the *Varroa* mite, when examination of deformed (symptomatic) bees revealed high titres of an icosahedral single stranded RNA virus (Bailey, *et al.*, 1981). However, DWV was not seen as a major issue by beekeepers or

researchers and symptomatic outbreaks were very rare. DWV was rarely present at levels high enough to be detectable by the techniques of the time. The honey bee research community is currently undecided as to the prevalence of DWV in honey bee colonies prior to the introduction of *Varroa*. Sensitive molecular techniques have revealed that low level, asymptomatic DWV infection can be detected in colonies from *Varroa* naive areas, such as those on Colonsay island (an island in the Inner Hebrides of Scotland, currently one of the few remaining *Varroa* free areas of Europe) and those in Northern Scandinavia, beyond the expansion front of *Varroa* (Yue and Genersch, 2005; de Miranda and Fries, 2008; Ryabov, *et al.*, 2014b). However, samples from Australia (the last large land mass to remain free of the parasite) have been shown to be DWV free (Roberts, *et al.*, 2017). Whether the virus could have been introduced by beekeeping practices in to these European samples, or whether it was widely present in the area, forming a persistent, asymptomatic infection, the fact remains that in the absence of *Varroa* the virus is capable of establishing a covert, asymptomatic infection.

DWV replication has also been identified in bumble bees (*Bombus pascuorum* and *Bombus terrestris*) and in a number of other ‘hive attendant’ arthropods, including the European earwig (*Forficula auricularia*) and the German cockroach (*Blattella germanica*) (Genersch, *et al.*, 2006; Levitt, *et al.*, 2013). These species are not vulnerable to *Varroa* parasitism, so they are thought to become infected through direct association with honey bee colonies, and possible ingestion of contaminated food or pollen (Levitt, *et al.*, 2013).

1.7 Deformed Wing Virus – pathology and virulence.

DWV is extremely widespread in *A. mellifera* colonies. For example, it was found to be present in 95% of British apiaries in a survey conducted by the National Bee Unit (G. Budge, National Bee Unit, unpublished data). In the absence of *Varroa*, DWV

infection usually persists at a very low level and does not result in visible symptoms or have any apparent negative impact on host fitness. However, for reasons that are still not fully understood, the transmission of DWV by *Varroa* to the developing pupae causes elevated virus levels and characteristic symptoms of atrophied wings, stunted abdominal development and cognitive/learning impairments (de Miranda and Genersch, 2010). Bees with high levels of DWV also have significantly shortened lifespans (Dainat, *et al.*, 2012a). *Varroa* infestation and the subsequent increase in DWV levels and symptomatic individuals within a hive have severe implications for the ability of a colony to gather stores, nurse larvae and, ultimately, survive overwinter. Large scale sampling studies of honey bee colonies across the UK and Europe have repeatedly reported that DWV is the pathogen most frequently associated with elevated *Varroa* loads, weakened colonies and increased overwintering losses and that high levels of DWV, particularly in the autumn when the colony is preparing for overwintering, is strongly correlated with colony loss (Highfield, *et al.*, 2009; Berthoud, *et al.*, 2010; Genersch, *et al.*, 2010; Budge, *et al.*, 2015)



Figure 1.7 An adult worker bee suffering from DWV. Symptomatic DWV infected honey bees have deformed wings, stunted abdomen, impaired learning capacities, reduced foraging ability and reduced longevity. Photo credit, Prof. D Evans.

In work recently published by our research group, we have demonstrated that a single virulent hybrid form of DWV accumulates to high levels in pupae upon which mites have fed. This recombinant virus is characterized as having DWV-like sequences for non-structural genes and VDV-1 like capsid protein genes (Ryabov, *et al.*, 2014a). In a significant proportion (approximately 70%) of pupae exposed to *Varroa*, the delivery of DWV directly into the hemolymph by the feeding mite results in the amplification of near clonal population of DWV-like virus to high levels (i.e. comparable to DWV levels found in symptomatic adult bees). Any pupae in the hive that have been unexposed to *Varroa*, along with the remaining 30% of the exposed individuals, have very low (asymptomatic) levels of DWV like viruses with a much greater diversity in virus population (Ryabov, *et al.*, 2014b). This pattern – increase in the level of DWV with a corresponding decrease in viral sequence diversity upon exposure to *Varroa* – was recorded on a much larger scale in a study that looked at alterations in the DWV population upon the introduction of *Varroa* to Hawaii (Martin, *et al.*, 2012).

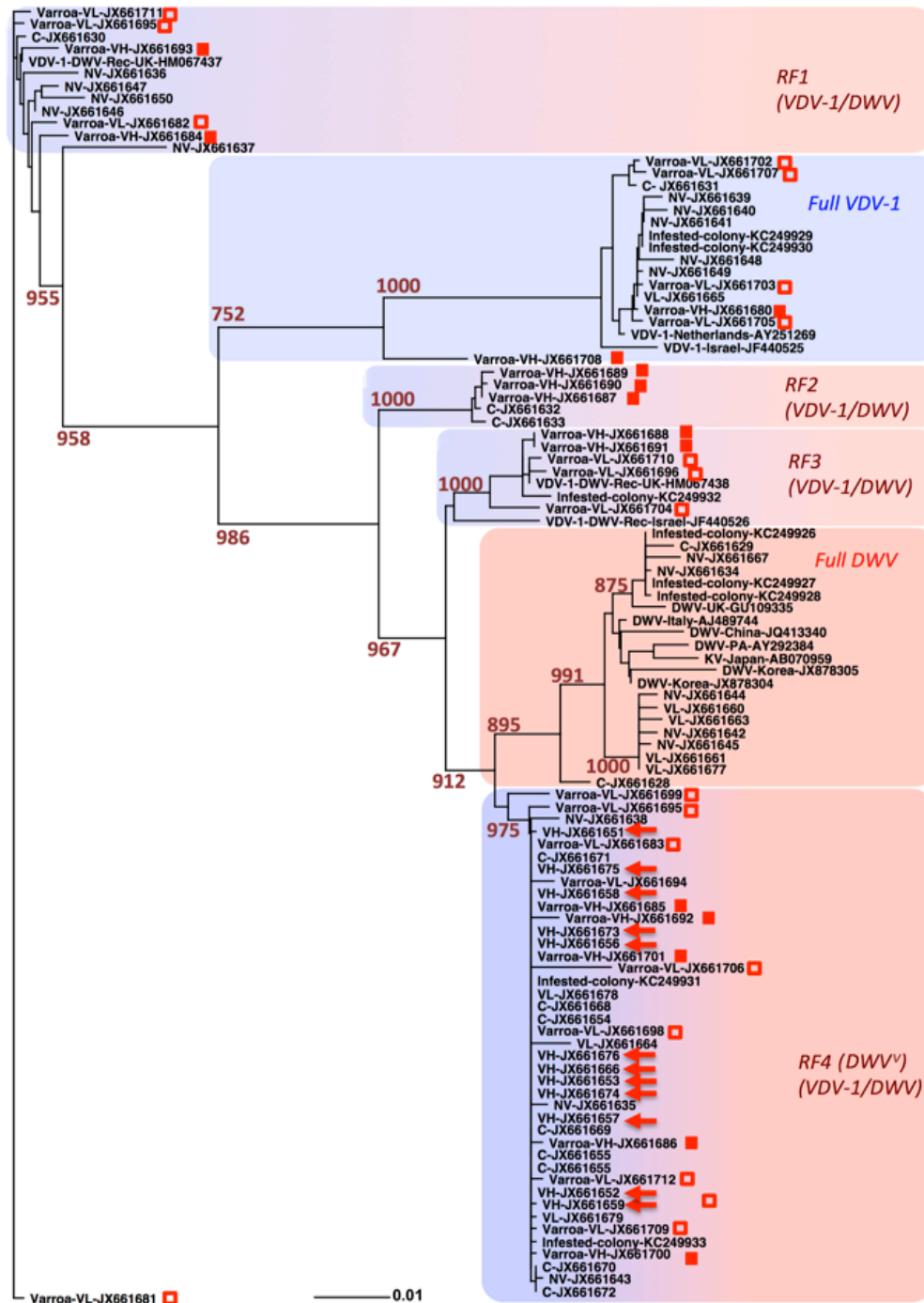


Figure 1.8 Phylogenetic analysis of the central region of DWV-like virus genome, figure from (Ryabov, et al., 2014b). Sequences derived from *Varroa* parasitized individual honeybee pupae with 'high' levels of DWV (determined by qPCR) are highlighted with arrows. All of these individuals had a near clonal population of DWV/VDV-1 recombinant virus, also shown by high-throughput sequencing of the honeybee small RNA libraries, see (Ryabov, et al., 2014b).

The research described above allows us to look at DWV-like viruses as a quasispecies, a rapidly evolving group of closely related RNA viruses (Domingo and Holland, 1997; Luring and Andino, 2010). DWV and VDV-1 can each be seen as the master variants, with inaccurate RNA replication and recombination leading to a swarm of variants. This theory is described in (Mordecai, *et al.*, 2016a), who go on to look at the predominant virus present in an isolated apiary of bees that appear to be able to withstand long term high *Varroa* levels and the subsequent elevated DWV-like virus levels. These bees were found to be infected with predominantly VDV-like viruses (named ‘DWV type B’ by Mordecai *et al.*) and they suggest that a prior infection with the ‘non-lethal’ type B variant prevents the type A (full length DWV-like) viruses establishing and leading to colony death. This interesting observation has not yet been recorded elsewhere, but it gives further weight to the theory that virus strain is closely linked to the route of transmission and virulence of DWV-like viruses.

The complexity of the system (and need for further research) is highlighted by the recent publication (McMahon, *et al.*, 2016b) in which, laboratory and field studies are used to demonstrate that an emerging DWV genotype (DWV-B, or VDV-like viruses) is more virulent than the established DWV genotype (DWV-A or DWV-like) and is widespread in the UK. The paper also shows that colonies infected with DWV-B collapse sooner than colonies infected with DWV-A. This result appears contradictory to the Mordecai paper, indicating that this complex system is not yet fully described by current theories and that more research is required.

1.8 The Honey Bee Transcriptional Response to *Varroa* parasitism and to DWV infection.

The honey bee response to DWV infection is not yet fully understood. One of the main strands of research has focused on revealing more about the honey bee anti-viral response, and how this is modulated by DWV infection and by *Varroa* parasitism. It is also hoped that investigation of the changes in gene expression during DWV infection may reveal more about the mechanisms by which the virus causes disease symptoms in honey bees. Here, it is useful to look at the state of current knowledge in three fundamental areas; the honey bee immune response to *Varroa* parasitism, the honey bee immune response to DWV infection and the honey bee developmental response to DWV infection.

1.8.1. The Honey Bee Immune Response to *Varroa* parasitism and to DWV infection.

Our current knowledge of the immune defence pathways in honey bees is largely based on identification of honey bee orthologs to better known and characterized genes from mosquito and *Drosophila* (Weinstock, *et al.*, 2006). This use of comparative genomics has revealed new information about honey bee immune gene pathways, but it is important to note that eusocial Hymenoptera, including *A. mellifera*, diverged from the Diptera (which contains *Drosophila* and mosquitoes) over 300 million years ago (Dearden, *et al.*, 2006; Evans, *et al.*, 2006). A comprehensive review of the insect immune pathways which have been revealed in honey bees is provided by (Brutscher, *et al.*, 2015).

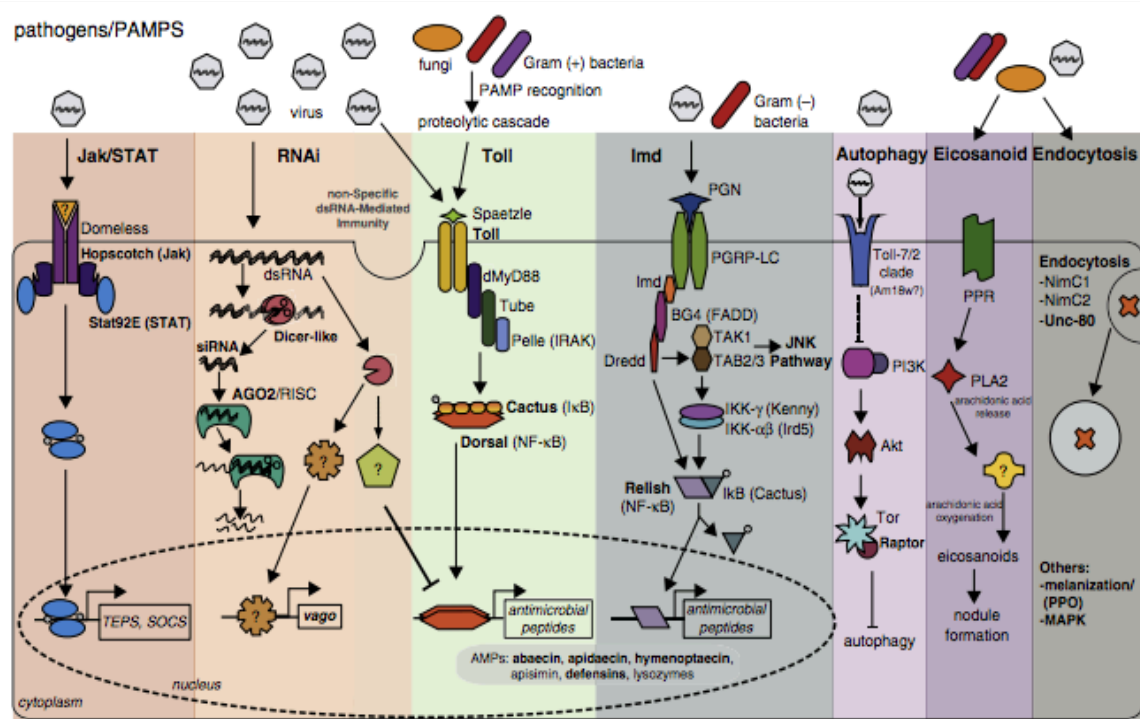


Figure 1.9 Honey bee immune pathways, with the genes that have been implicated in antiviral responses highlighted in bold text. The main immune pathways present in honey bees are; RNAi, Jak/Stat, Toll;NFκB, JNK, and MAPK. This figure has been adapted from (Brutscher, et al., 2015), a comprehensive review of antiviral defence mechanisms in honey bees.

Many ectoparasites, especially ticks (Wikel and Bergman, 1997; Schoeler and Wikel, 2001) immune-suppress their hosts to facilitate extended feeding, and these immunocompromised hosts then become more vulnerable to infectious diseases (Titus and Ribeiro, 1988; Schwan, et al., 1995; Edwards, et al., 1998). This knowledge has prompted research into how *Varroa* parasitism affects honey bees, in an attempt to establish whether immune pathways are compromised in *Varroa* exposed individuals, potentially explaining the close association between *Varroa* mites and honey bee viruses, as parasitized bees would be more vulnerable to infection. However, gene expression analysis of *Varroa* parasitized honey bees has produced contradictory results about the impact that *Varroa* feeding has on the immune response of its host. The first study published on the topic reported that *Varroa* exposed, newly emerged, honey bees

had reduced expression of genes encoding antimicrobial peptides (AMPs - abaecin, defensin, and hymenoptaecin) and immunity-related enzymes (phenol oxidase and lysozyme) compared to the expression levels in *Varroa* naïve bees when challenged with heat inactivated *E.coli* (Yang and Cox-Foster, 2005). Similarly Gregory et al. (2005) found that bees that had been parasitized by *Varroa* also had lower levels of AMP gene expression, although there was no evidence that these immunosuppressed bees were more vulnerable to infection, with no correlation between bacterial or viral infection and AMP expression level being established (Gregory, *et al.*, 2005). In contrast, subsequent publications reported that, although there were some subtle differences in gene expression between *Varroa* exposed and *Varroa* naïve bees in genes associated with neuronal development and metabolic activities, there was no difference in the expression of immune related genes between the two groups (Navajas, *et al.*, 2008; Kuster, *et al.*, 2014). Microarray analysis conducted by our research group (Ryabov, *et al.*, 2014a) found some perturbation of immune related genes from the Toll pathway and significantly enhanced expression of the honeybee orthologue of Vago (GB10896), a secreted protein that is upregulated in mosquitos and *drosophila* during the RNAi response to viral infections (Deddouche, *et al.*, 2008; Paradkar, *et al.*, 2012). However, the contrasts between the treatment groups did not reveal any immune pathways that were consistently up or down regulated, and the majority of the previously implicated AMPs appeared to be unaffected by *Varroa* parasitism.

Comparative genomics indicates that honey bees possess the same putative antiviral defence pathways as *Drosophila* and mosquito, notably the Jak/STAT, Toll and Imd signalling pathways, RNA interference, endocytosis, as well as MAPK and melanisation reactions (Fig. 1.8). RNA interference (RNAi) is the main route of antiviral defence in both fruit flies and in mosquitos (Ding, 2010), and has therefore been of particular interest to researchers investigating how honey bees respond to viral infections. RNAi is a sequence specific, post-transcriptional gene silencing mechanism that is elicited by dsRNA. Laboratory and field experiments have shown that feeding bees with virus specific dsRNA, to trigger the RNAi response, provides some protection from IAPV and

DWV infections (Maori, *et al.*, 2009; Hunter, *et al.*, 2010; Desai, *et al.*, 2012). It has also been demonstrated that administration of non-specific dsRNA, regardless of sequence, can suppress experimental infections of Sindbis virus expressing enhanced green fluorescent protein (SINV-GFP) (Flenniken and Andino, 2013; Brutscher, *et al.*, 2017). Experimental infection with SINV-GFP also revealed differential expression of genes involved in RNAi, Toll, Imd, and JAK-STAT pathways, and *in vivo* knockdown of *dicer* (RNAi pathway) and *cyclin dependent kinase* (uncharacterized) lead to increased virus levels (Brutscher, *et al.*, 2017).

No consensus has yet been reached in the published literature on the role of the putative bee antiviral defence pathways in relation to parasitism by *Varroa* and its associated viruses. The Toll pathway component dorsal-1A was implicated in limiting DWV infections in honey bees (Nazzi, *et al.*, 2012), with downregulation of dorsal-1A expression leading to increased DWV loads. However, the same study found no alteration in expression of components of the RNAi pathway in heavily *Varroa* infested, DWV infected bees. Similarly, Ryabov *et al.* (2014) observed that some proposed components of the Toll signalling pathway were differentially expressed in response to *Varroa* parasitism, but the lack of expression of the AMPs that are the end products of these pathways suggested that Toll and Imd activation had not been triggered. Moreover, no significant changes in expression of genes associated with the RNAi response (such as Argonaute and Dicer) were observed in their analysis of *Varroa* exposed, DWV infected honey bees. In contrast, in their research into the immune responses of *Varroa* exposed pupae, (Kuster, *et al.*, 2014) found that expression of *defensin*, *apidaecin*, *abaecin*, *hymenoptaecin*, *PPOact*, *PGRPs*, and *relish* increased with increasing DWV load, implying that the Imd and Toll pathways are central in the honey bee immune response to DWV infection.

One concern faced by all of these publications, and a possible explanation for the inconsistencies in results, is that it is difficult to separate the effects of *Varroa*

parasitism (with its associated wounding, introduction of non-viral pathogens and possible immune suppression) from the effects of *Varroa*-mediated viral infections, since the majority of *Varroa* exposed bees also carry a high viral load (Gregorc, *et al.*, 2012). To date, there has been little work published that separates the effect on honey bees of *Varroa* feeding on its own from the impact of *Varroa*-mediated DWV infection. The *in vitro* collection of *Varroa* saliva has allowed a preliminary biochemical characterization of the secretions, with 15 distinct proteins present in the saliva (Richards, *et al.*, 2011). The *Varroa* saliva was also shown to impair the function of *Lacanobia oleracea* (Lepidoptera, Noctuidae) haemocytes in culture, indicating that these salivary proteins may facilitate the suppression haemocyte-mediated wound healing (Richards, *et al.*, 2011). Another possible explanation for the diverse results found by different research groups could be that different life stages of honey bees were sampled, and the expression of immune related genes is predicted to be very transient and dependent on developmental stage (Zaobidna, *et al.*, 2017). Detailed analysis of the expression of 14 genes in the Toll pathway, including effector genes of AMPs, in response to *Varroa* exposure in larvae, prepupae and pupae, found that the immune response was most pronounced in larvae, with a significant increase in the expression of 10 immune related genes, including defensin-1 and defensin-2, being observed in *Varroa* exposed bees, relative to non-infested individuals (Zaobidna, *et al.*, 2017).

The difficulty in distinguishing between the immune response to *Varroa* feeding and viral infections resulted in the two being classed together in a recent meta-analysis of multiple transcriptome data sets (Doublet, *et al.*, 2017). The analysis found 167 genes that were consistently differentially expressed in response to *Varroa*/virus treatments (79 down-regulated and 88 up-regulated). The transcriptional response to *Varroa*/virus treatments included in the analysis was distinguished by the differential expression of genes from the Toll (tube and def-2) and Imd pathways (iap2 and relish). Doublet *et al.* note that they did not observe any differential expression in genes associated with the RNAi pathway, they suggest that this may be due to the transient nature of the RNAi

response, meaning that the expression of these genes may be missed in meta-analysis incorporating data from honey bees of different ages and times post infection.

1.8.2. The Honey Bee Developmental Response to *Varroa* parasitism and to DWV infection.

The mechanism by which *Varroa* and DWV cause disease symptoms in infected bees is not well understood (de Miranda and Genersch, 2010). A ‘successful’ DWV infection has been shown to have a specific and predictable effect on a developing honey bee pupae, with bees injected with DWV going on to develop characteristic symptoms of atrophied wings, stunted abdominal development and cognitive/learning impairments (Yue, *et al.*, 2007; de Miranda and Genersch, 2010; Moeckel, *et al.*, 2011). The transition from the bee pupa to the adult stage is a highly dynamic, programmed process that entails remodelling of tissues and growth of new organs. To date, nothing has been published about how this process is affected at the molecular level by *Varroa* parasitism and DWV infection, but a recent microarray analysis of gene expression in honey bee pupae, controlling tightly for age of pupae and parasite load, found differential expression of genes associated with morphogenesis, including a number of ecdysone response genes, known to be controlled by the ecdysone- ultraspiracle receptor complex in *D. melanogaster* (Gauhar, *et al.*, 2009) and a strong effect of *Varroa* parasitism on the expression of honeybee homeobox genes (>70% of homeobox genes were differentially expressed in response to *Varroa* mite burden) (Chandler *et al.*, unpublished data)

Insect morphological development is governed by a suite of evolutionarily conserved homeodomain proteins, transcription factors and co-factors which regulate cell fate and cell type gene expression to determine insect segment morphology and system development. (Walldorf, *et al.*, 2000; Ladam and Sagerstrom, 2014). The homeobox is a sequence of DNA coding for 60 amino acids that was originally discovered in the

genome of the fruit fly *D. melanogaster*, and has now been characterised in all three kingdoms of multi-cellular organisms. Homeobox containing genes are translated into DNA binding proteins which are crucial in the regulation of gene expression in morphogenesis and cell differentiation (Mark, *et al.*, 1997). Very little is known about the functions of homeodomain containing genes in honey bees, but databases such as HomeoDB allow us to see the homologs from model organisms such as fruit flies (Zhong, *et al.*, 2008; Zhong and Holland, 2011).

The microarray results reported above are intriguing as they are the first time that developmental genes have been found to be overrepresented in the differentially expressed gene set from *Varroa* exposed, DWV infected honey bees. This disturbance in gene expression may help to explain the morphological deformities that occur in parasitized bee pupae, and they provide a platform for more detailed, mechanistic studies of the bee-virus-*Varroa* interaction. If our observations are correct, they also raise questions of whether the alteration in host phenotype is: (i) beneficial to *Varroa* and / or DWV (and therefore subject to natural selection); (ii) a response of the bee to limit the damaging effects of parasitism (ditto); or (iii) is simply a side-effect of the infection process (Schmid-Hempel, 2011). Further work is necessary to establish whether this disturbance in developmental gene pathways is a consistent response in genetically diverse honey bees, and to establish if the perturbation is due to *Varroa* parasitism or to DWV infection.

1.9. Project outline, Hypothesis and Aims

The spread of the parasitic mite, *Varroa destructor* (and the RNA viruses that it transmits between honeybees), is closely linked to increased overwintering losses and declining honeybee health. Deformed wing virus (DWV) is widespread in honeybees and usually present as a low level, asymptomatic infection. However, the transmission

of DWV by *Varroa* to developing pupae causes markedly elevated levels of a near clonal virus population, leading to characteristic developmental abnormalities. At present, there are significant gaps in understanding about the pathology of Varroa-mediated DWV infections, and this is preventing the development of new interventions to effectively mitigate the effects of DWV and *Varroa* on honey bee populations.

The aim of this project is to generate new knowledge on the interactions between honeybees, *Varroa* and Deformed Wing Virus to underpin the development of improved management options for beekeepers. Three different research questions were addressed.

1. How does DWV transmission route influence virus accumulation, localization and diversity?

We know that the mode of DWV transmission plays a crucial role in determining the outcome for infected honey bees. Individuals infected via *Varroa* feeding are more likely to go on to have a high viral load than those infected orally or vertically (Yue, *et al.*, 2007; Moeckel, *et al.*, 2011). We hypothesise that DWV is more infectious when transmitted by Varroa because it is injected directly into the haemolymph, bypassing the host's immune defences and gaining access to a wider range of tissues. We have tested this hypothesis through experimental infection of honey bees with DWV, followed by detailed tracking of the progress of infection via qPCR and a novel RNA-FISH imaging technique.

2. How does the spread of the *Varroa* mite alter the prevalence and population diversity of DWV like viruses?

It has been widely reported that the spread of the *Varroa* mite has coincided with the emergence of a highly virulent strain of DWV, which may have a selected advantage when transmitted by the mite (Martin, *et al.*, 2012; Ryabov, *et al.*, 2014a; McMahon, *et al.*, 2016b; Mordecai, *et al.*, 2016a). However, there is currently disagreement in the published literature with regard to the exact genome of this virulent strain, with some groups pointing towards DWV-like viruses being more virulent and associated with *Varroa* parasitism (Mordecai, *et al.*, 2016a; Brettell, *et al.*, 2017), some identifying VDV-like strains (McMahon, *et al.*, 2016a) and others pointing towards a recombinant VDV/DWV viral sequence (Ryabov, *et al.*, 2014a). Two repeated sampling experiments have been conducted to try and resolve these inconsistencies, looking at the change in DWV population at the forefront of the *Varroa* invasion in the UK, and attempting to track any changes in the predominant strain of the virus over a single season in a heavily *Varroa* infested honey bee colony.

3. How are *Varroa destructor* and Deformed Wing Virus responsible for developmental abnormalities in honeybees.

The mechanism by which *Varroa* and DWV causes disease in individual bees is not well understood (de Miranda & Genersch, 2010). We propose that DWV infection during crucial stages of honey bee pupation and metamorphosis causes disturbances to the expression of developmental gene pathways, including ecdysone receptor signalling and homeobox developmental genes, leading to the characteristic DWV symptoms. This hypothesis is tested through comprehensive measuring of the expression of key developmental genes in honey bee pupae infected with DWV, both via *Varroa* feeding and experimental inoculation.

Chapter 2. Materials and Methods

This chapter gives details of the main techniques and protocols used throughout the research chapters of this thesis. Where alterations to standard techniques have been employed full particulars are given. Details specific to individual experiments (sample numbers, replicates, sampling dates etc.) have been given in the relevant chapters.

2.1 Beekeeping

A number of honey bee (*Apis mellifera*) colonies were maintained for the duration of this project to provide insects for direct sampling and insect manipulation experiments. Details are provided here of the colonies used for sampling in the 2015, 2016 and 2017 seasons. The bees used for this work have not been genotyped, but they are thought to be a mixture of subspecies, as is common in non-commercial apiaries in the UK – with the most common subspecies being *Apis mellifera mellifera* (the European dark honey bee) and *Apis mellifera lingustica* (The Italian honey bee) (Lodesani and Costa, 2003; Carreck, 2008). Where further detail on the heritage of individual colonies is known, it is given below.

Colonies were all inspected weekly during the beekeeping season (April to September) to check colony health and to control for swarming. At each inspection, the presence of the queen, the number of frames of brood and stores and the general health and temperament of the bees was recorded. *Varroa* population monitoring was also carried out regularly (method described below). If the colony was strong or looked to be preparing to swarm, a split was made using the Pagden method of swarm control, as described in (NationalBeeUnit, 2017). All colonies were fed with Ambrosia fondant (manufacturer Nordzucker) during September to encourage them to build up stores for the winter.

2015

Two independent colonies were maintained at apiary A, on the University of Warwick campus, in Coventry. Beebase Apiary co-ordinates SP293755. The colonies were established on the site during 2014 and were first generation descendants of colonies imported from Colonsay, Scotland (subspecies *Apis mellifera mellifera*, <http://www.snhbs.scot/colonsay/>) hybridised with local honey bees. *Varroa* mite numbers and DWV levels were measured at the start of the season, one colony (Blue) underwent no *Varroa* control measures whilst the other colony (White) was treated with vaporised oxalylic acid (<http://theapiarist.org/sublimox/>)

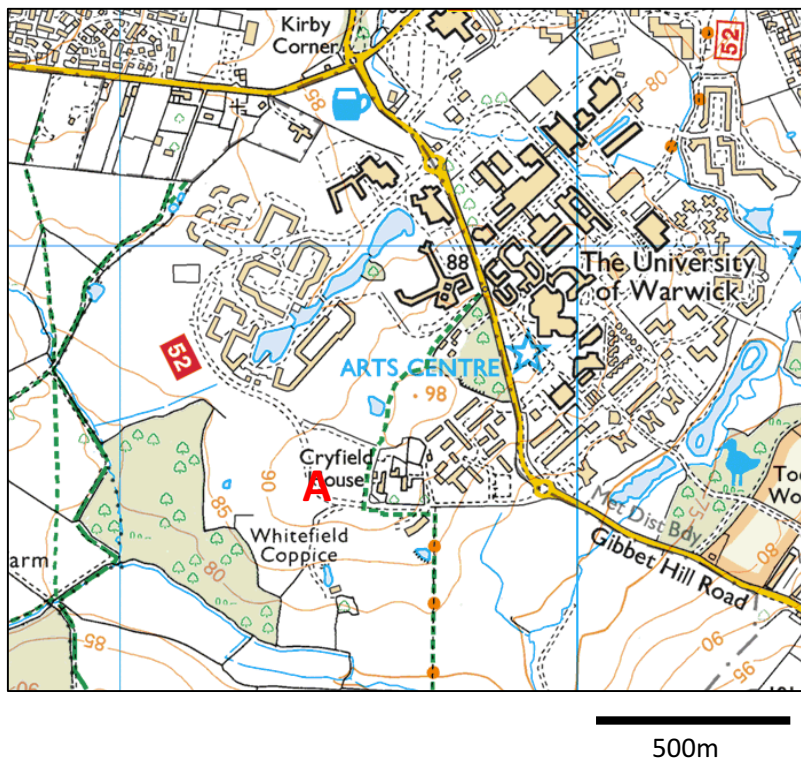


Figure 2.1. The location of the experimental apiary A in 2015 on the University of Warwick main campus.

2016 and 2017 seasons

Apiary coordinates SP270571 and SP267566

2016 - After a site move, the beekeeping for 2016 and 2017 seasons took place at The University of Warwick, Wellesbourne site, with two separate apiaries on site. Site B, 'Townsend' housed two well established experimental colonies (thought to be hybrid *A. mellifera mellifera* and *A. mellifera lingustica* subspecies) along with the surviving 'White' colony from the 2015 season. One colony on this site was lost to swarming during the season.

Site C 'Top' was a newly established apiary containing four colonies, purchased as 5 frame nuclei with overwintered Buckfast queens from BS Honeybees (<https://www.bshoneybees.co.uk/>) in May 2017. Two of these colonies became well established and were used for sampling throughout the season, whilst two failed to thrive due to poorly mated queens and were therefore not used for any experimental work.

2017 – Townsend apiary (B), two well established colonies made it through the winter and one was divided as swarm control early in the season. These colonies were supplemented with a collected swarm from the Coventry area to give an apiary of 4 colonies for the 2017 season.

Apiary C began the season with just two of the Buckfast colonies from 2016. In the spring 4 new 5 frame nuclei were purchased from Paynes Southdown Bee Farms (West Sussex) and added to the apiary.

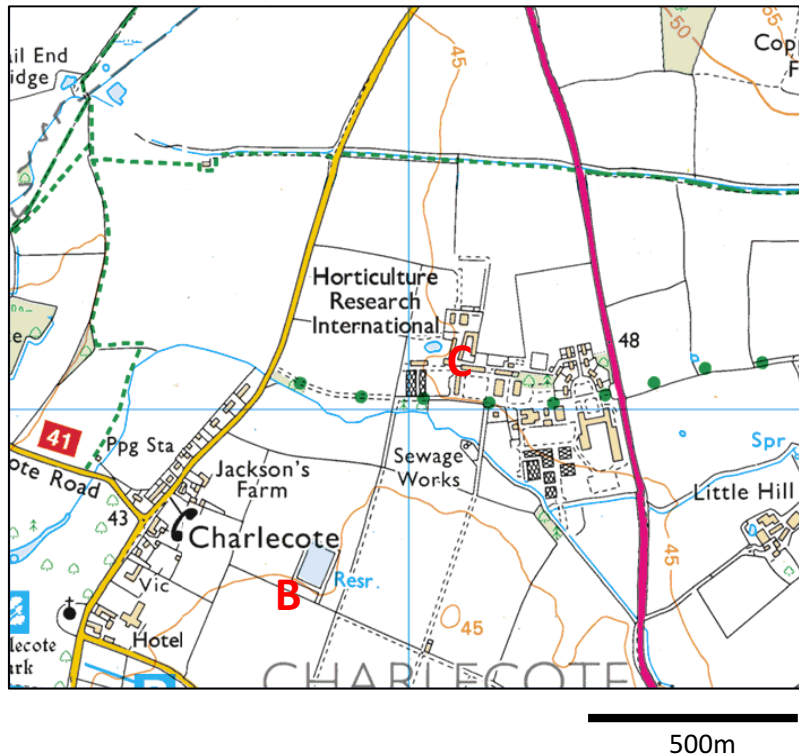


Figure 2.2. The locations of experimental apiaries B and C on Wellesbourne campus, used for experiments in 2016 and 2017.

2.1.1 *Varroa* population monitoring

Varroa levels were regularly monitored across all colonies using the Natural Mite Mortality method, as described by the National Bee Unit:

- 1) Attach a sheet of A3 paper, coated in petroleum jelly to trap falling mites, to the base board of the hive and insert under the open mesh floor.
- 2) During summer collect colony debris for 7 days;
- 3) During winter collect debris for two weeks;
- 4) Count the total number of mites collected, divide this figure by the number of days the sample was taken over to give a daily mite fall number;

5) Multiply the daily mite fall figure by one of the following. Winter i.e. November to February x400 Summer i.e. May to August x30 March, April, September and October x100 (These periods are approximate only)

(www.nationalbeeunit.com/downloadDocument.cfm?id=199)

2.2 Isolation of virus particles from honey bees

There is currently no functional reverse genetic system or infectious full-length clone available for DWV infection research. Therefore, virus particles must be purified from naturally infected honey bee tissue for use in injection and feeding experiments. DWV-like viruses can be purified from almost any honey bee tissue but the greatest yield comes from symptomatic (deformed wings, stunted abdomen) adult bees or heavily *Varroa*-infested pupae. For the experiments described in this thesis, a virus preparation was generated from mite-exposed pupae, taken from a high *Varroa* colony on Wellesbourne Campus in May 2015. The exact protocol used was developed in the lab and remains unpublished, so full details of the protocol are given here:

1. Homogenize 5g of insect tissue, frozen or live, with 35 mL sodium phosphate buffer 100mM, pH 7.4 (NaP buffer) supplemented with 0.05% of Tween 20 using a pestle and mortar.
2. Centrifuge for 12 min in a high-speed centrifuge (22848 x g Relative Centrifugal Force (RCF), 4°C)
3. Carefully remove and retain the supernatant, leaving the fat layer (top) and the un-homogenized tissue (bottom).
4. Layer the cleared supernatant over a 20% sucrose cushion (2.5 mL, 20% sucrose in NaP buffer) in 30 mL ultracentrifuge tube, Beckman SW28 rotor. Top up with the NaP buffer so that liquid reaches 0.5 cm from the top of the tube.
5. Centrifuge at 28000 r.p.m (141000 x g RCF), 18°C, for 3h 30 min, pour off the supernatant and re-suspend the pellet in 2 mL of NaP buffer, leave overnight at 4°C to ensure complete resuspension of the virus particles.

7. Set up a discontinuous caesium chloride (CsCl) density gradient in a 35 mL ultracentrifuge tube, Beckman SW28 rotor, by layering from the bottom 5 mL aliquots of CsCl with the following densities 1.6 g/cm³, 1.5 g/cm³, 1.4 g/cm³, 1.3 g/cm³, and 1.2 g/cm³. The CsCl solutions are prepared using saturated CsCl (1.9 g/cm³ in dH₂O) mixed with NaP buffer in varying proportions to achieve required density. Carefully layer the re-suspended sucrose cushion pellet containing the virus particles over the CsCl gradient after mixed with enough buffer to reach 0.5 cm from the top of the tube.
8. Centrifuge for 18 hr, 28000 r.p.m. (141000 x g RCF) at 4°C, Beckman SW28 rotor.
9. Carefully collect separate 1.5 ml fractions, starting from the top.
10. Weigh each 1.5 mL fraction to determine density, collect fractions with density 1.30 to 1.40 g/cm³, pool them together and dilute 5 to 10-fold with NaP buffer.
11. Centrifuge the diluted fractions for 3hr, at 4°, 28,000rpm (141000 x g RCF), SW28 Beckman bucket rotor.
12. Immediately remove supernatant and re-suspend the pellet with 200 µL 0.1 M NaP buffer, pH 7.4. Note that pellet could be invisible. Store at -80°C.

2.3 *In vitro* larval maintenance, for larval feeding experiments.

In order to investigate the interaction between orally administered DWV and developing honey bees, a method that allows young (3 days from laying) larvae to be taken from a colony and fed and maintained in the lab was required. The technique is described in full in the COLOSS BEEBOOK (Crailsheim, *et al.*, 2013) but details of the conditions used for the experiments described in this report are given here.

2.3.1 Larval feeding protocol

Three-day old honeybee larvae were used for larval maintenance experiments (from 2015 White, low *Varroa* colony). Correct larval age was ensured by placing the empty

brood frame into the center of the brood box 5 days prior to sampling, and then through careful checking of the size and appearance of each individual selected, using the COLOSS Beebook larval age standard images (Figure 2.1) (Human, *et al.*, 2013). The larvae were gently washed out of their frame with warm (30°C), sterile water. They were quickly and gently moved into 6 well plates (Falcon® 6 Well Clear Flat Bottom TC-Treated Multiwell Cell Culture Plate), 6 larvae per well, on top of sterile polyester disks (Anti static polyester lining fabric, Fabric UK. Disks 3cm diameter) and 300 µl warm larval diet (6 g D-(+)- glucose, 6 g D-(-)- fructose, 1 g Yeast Extract, 37 ml ddH₂O, 50 g Royal Jelly (Wellbee-ing UK). Larvae were maintained in an incubator (Thermo Scientific, BBD 6220 CO₂ incubator, 33°C, 90% humidity) and each individual was fed approximately 50 µl warm (30°C, warmed in water bath) larval diet four times per day. Larvae were transferred into clean 6 well plates each morning, and any dead individuals were counted and removed. Once the larvae reached pupation age, on day 5 of the feeding experiment, they were gently rolled on filter paper to remove any adherent food or waste products and placed into petri dishes containing clean filter paper. Pupae require no feeding and were allowed to develop at 33°C, 80% humidity (Thermo Scientific, BBD 6220 CO₂ incubator).



Figure 2.3 *In vitro* larval maintenance, for larval feeding experiments. Larvae were reared in flat well plates, on disks of anti static polyester lining fabric. Maintained in an incubator at 33°C, 90% humidity and fed 50 µl warmed larval diet 4 times per day.

2.4. Honeybee injection for *in vitro* administration of DWV.

Individual pupae or newly emerged adult bees were injected with DWV directly into the haemolymph to mimic exposure to DWV through *Varroa* feeding.

2.4.1 Pupae injections Protocol.

A frame of capped brood was removed from the colony and brought quickly to the lab in an insulated carrier to prevent chilling. Brood cells were uncapped using forceps and individual pupae were gently removed and inspected for *Varroa* mites. Once the desired number of *Varroa* free pupae of the appropriate age had been collected, they were injected using a 25 µl Hamilton syringe with a 26s gauge needle, along with a repeating dispenser to ensure that a 0.5 µl dose was administered with each injection (Hamilton Repeat Dispenser, model PB-600). The syringe was held in a fixed position on staging (Prior, England), with the insect being brought towards the needle and penetrated in the side of the abdomen, between the developing tergites. The needle was inserted parallel to the tergite, avoiding the gut and entering the into the haemocoel. Individuals were injected with either sterile phosphate-buffered saline (PBS) or DWV virus prep (Wellesbourne 2015 VP, from symptomatic DWV individuals) diluted to the desired concentration in PBS. Injected pupae were placed into petri dishes containing filter paper and stored in a sterile, humidity controlled incubator (Thermo Scientific, BBD 6220 CO₂ incubator) at 33°C, 80% humidity until sampling. An inspection 12 hrs after injection allowed any individuals damaged by handling or injection to be excluded from the experiment.



Figure 2.4. Honeybee injection for in vitro administration of DWV.

2.4.2 Adult injections.

Adult honey bee injections were all carried out using newly emerged, *Varroa* free individuals, collected from a single colony. A frame of capped brood was removed from the colony, after checking that the majority of the brood was at the ‘pupal moult’ or ‘resting adult’ stage of development, 11 to 12 days post capping (see Figure 2.4). Individuals were allowed to emerge naturally in the lab, under observation, any with *Varroa* (on the bee or in the cell) were discarded, and all *Varroa* free bees were collected in disposable flight cages with a diet of 50% sucrose solution, as described in (Evans, *et al.*, 2009). The disposable flight cages or ‘bee-cups’ were made up of a disposable plastic half pint tumbler (Nisbets Catering Equipment) paired with a plastic Petri dish (84 mm diameter, Sarstedt) and a plastic scintillation vial (20 ml, Sarstedt). Bee-cups were constructed as per Evans, *et al*, 2009 (Figure 2.3).

Once the desired number of *Varroa* free honey bees of the appropriate age had been collected, they were injected using a 25 μ l Hamilton syringe with a 26s gauge needle, along with a repeating dispenser to ensure that a 0.5 μ l dose was administered with

each injection. Injections were always carried out within 12 hours of emergence, whilst the cuticle was soft and the bees were still unable to sting. The syringe was held in a fixed position on staging (Prior, England), with the insect being brought towards the needle (held still by the wings) and penetrated in the side of the abdomen, between the cuticle segments (tergites), into the haemocoel. Individuals were injected with either sterile phosphate-buffered saline (PBS) or DWV virus prep (Wellesbourne 2015 VP, from symptomatic DWV individuals) diluted to the desired concentration in PBS. The injected bees were kept in treatment groups within disposable flight cages with a diet of 50% sucrose solution in a humidity controlled incubator (Thermo Scientific, BBD 6220 CO₂ incubator) at 33°C, 80% humidity until sampling (Evans, *et al.*, 2009).



Figure 2.5 Disposable flight cage for injected adult honey bees, designed following instructions in (Evans, *et al.*, 2009). Injected bees were kept in treatment groups within disposable flight cages with a diet of 50% sucrose solution in a humidity controlled incubator (Thermo Scientific, BBD 6220 CO₂ incubator) at 33°C, 80% humidity until sampling

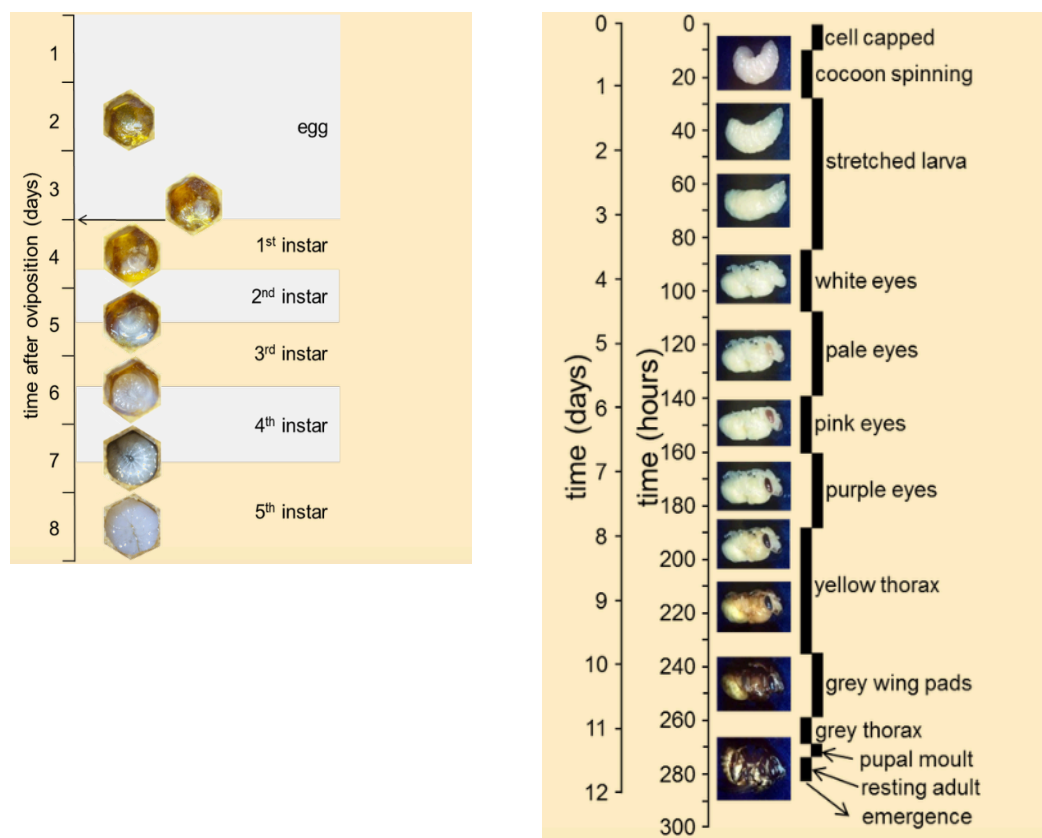


Figure 2.6 Standardised larval and pupal ageing charts, as used in all experiments. This figure has been adapted from the COLOSS Beebook (Human, et al., 2013)

2.5. Sample processing for molecular analysis.

2.5.1. Insect sampling

Live insects to be sampled were flash frozen in liquid nitrogen and stored in individual 1.5 ml sample tubes (Eppendorf) at -80°C. For direct sampling of capped brood from colonies, brood frames (or excised sections) were returned to the laboratory in an insulated polystyrene container and then individual pupae were taken from cells, aged according to Figure 2.6, inspected for *Varroa* and then flash frozen in liquid nitrogen.

2.5.2. RNA Extraction and processing

All equipment (pestle and mortar, Eppendorf tubes, spatula) was chilled in liquid nitrogen prior to use. Frozen honey bee tissue was homogenized whilst submerged in liquid nitrogen. The powder was not allowed to thaw and was split into two chilled 1.5 ml Eppendorf tubes – one for downstream RNA extraction, the other stored at -80°C. RNA extraction was carried out using TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. Total RNA was re-suspended in 50 µl RNase free water and stored at -80°C.

2.5.3 RNA clean up

RNA concentration was determined using a Nanodrop spectrophotometer. 10 µg of total RNA was then treated with DNase I (NEB) according to manufacturer's protocol to remove any contaminating genomic DNA. Immediately after this reaction, the sample was further purified (and any DNase enzyme removed) using the RNA GeneJET kit (Thermo Scientific) following the RNA clean up protocol.

2.5.4. Reverse Transcription

The clean RNA was then used immediately for reverse transcription, creating cDNA for PCR or qPCR analysis. 10 µl of DNase I treated column-purified RNA was used in each reaction, along with random hexamer (N10) primer (100 µM), RNase Out (Invitrogen) and SuperScript II (Invitrogen) according to manufacturer's instructions. cDNA samples were diluted 1:5 in dH₂O and stored at -20°C.

2.6. DNA analysis

2.6.1 Nested PCR detection of DWV, VDV1 and their recombinants

Identification of the strains of DWV-like viruses, in particular highly virulent VDV-1-DWV recombinants, requires cloning and sequencing of cDNA fragments (Ryabov, *et al.*, 2014). The levels of DWV in asymptomatic bees may be not be sufficient for direct amplification of the cDNA fragments for further characterization. Nested PCR, which includes amplification of a fragment of cDNA in two stages, first with a pair of “outer primers” and then with the pair of “inner primers” located within the re-amplified region, allows amplification of low levels of template. In respect to DWV and VDV-1 infection, a highly characteristic region suitable for identification of individual strains of DWV, VDV-1 and VDV-1-DWV recombinants is 1.3 kb fragments at the border between the structural genes (CP) and non structural (NS) gene blocks (corresponding to nucleotides 4926–6255 of the DWV genome; GenBank accession No. AJ489744). The outer and inner primer sequences are shown in Appendix 1.

Step One – using flanking primers “Nested-outer-universal-DWV-4700-For” and “Nested-outer--universal-DWV-6700-Rev”

For a 50µl reaction volume:

<u>Component</u>	<u>Volume (µL)</u>
GoTaq Green Master Mix, 2X	25
Forward primer (100 µM)	1
Reverse primer (100 µM)	1
cDNA template (diluted 1:5 in dH ₂ O)	5
dH ₂ O	18

The components were mixed in a PCR tube and placed into the thermal cycler for the following: 95°C, 2 minutes, 15 cycles (95°C, 30 sec - 52°C, 1min - 72°C, 2min), 95°C, hold.

Step 2 – using DWV or VDV specific primer combinations. There are 4 possible inner primer combinations, allowing amplification of DWV, VDV-1 and VDV-1-DWV recombinants. For amplification of the non-recombinant DWV (“Nested-Inner-DWV-4900-For” and “Nested-Inner-DWV-6500-Rev”); non-recombinant VDV-1 (“Nested-Inner-VDV-4900-For” and “Nested-Inner-VDV-6300-Rev”), VDV-1 CP – DWV NS recombinants (“Nested-Inner-VDV-4900-For” and “Nested-Inner-DWV-6500-Rev”), and DWV CP – VDV-1 NS recombinants (“Nested-Inner-DWV-4900-For” and “Nested-Inner-VDV-4900-For”) though the DWV CP-VDV-1 NS recombinant has never been detected.

5 µl of the ‘Step 1’ reaction served as the template for each of these ‘Step 2’ mixes:

For a 50 µl reaction volume:

<u>Component</u>	<u>Volume (µL)</u>
GoTaq® Green Master Mix, 2X	25
Upstream primer (100 µM)	1
Upstream primer (100 µM)	1
Product from ‘Step 1’ at 95°C	5
dH ₂ O	18

The components were mixed in a PCR tube and placed into the thermal cycler for the following: 95°C, 2 min, 30 cycles (95°C, 30 sec - 52°C, 1min - 72°C, 2min), 72°C, 10 min.

The product of each ‘Step 2’ reaction was analysed by agarose gel electrophoresis (1% gel on TAE buffer, 100V for 30min, stain with ethidium bromide), to determine which strains of DWV-like viruses and which recombinants are present in the sample. Further information on virus identity for phylogenetic analysis can be obtained by

cloning these PCR products into a plasmid vector (e.g. pGemT- Easy, Promega) and sequencing the plasmid using the Sanger dideoxy method.

2.6.2. Real-time PCR quantification of DWV

Real time PCR (or qPCR) allows accurate and simple quantification of DWV-like viruses within individual bees. With qPCR, fluorescent dyes are used to label PCR products during thermal cycling and the accumulation of fluorescent products is measured in real time. In my work, I have used qPCR to quantify viral load in individual bees, normalized to a constitutively expressed housekeeping gene (actin). I have also used this technique to give an indication of the prevalence of DWV capsid and polymerase sequences, with primers designed to anneal to a single reference strain of DWV or VDV-1 from GenBank (DWV-PA [AY292384.1](#), VDV-1 [KC786222.1](#)) (Appendix 1)

For a 20 µl reaction volume mix:

<u>Component</u>	<u>Volume (µL)</u>
Brilliant III QPCR Master Mix, Low ROX	5
dH ₂ O	3.8
Forward Primer (100 µM)	0.1
Reverse Primer (100 µM)	0.1
cDNA template	1

Reaction conditions: 95°C – 3 min, 50 cycles (95°C – 10 sec, 60°C – 30 sec). All qPCR carried out using a Roche Lightcycler 480 machine.

For absolute quantification of viral copy number, serial dilutions of known amounts of template were included on the qPCR plate and their ct scores used to plot a standard curve. The ct scores of the individual samples were then transformed using the equation of the standard curve to give the concentration per well, which was then converted to molecule copy number per well using the known molecular weight of the standards.

2.7. Localisation of DWV in honey bees using Stellaris probes (RNA-FISH) and confocal microscopy

Individual honey bee samples were experimentally exposed to DWV and then fixed, embedded in paraffin, sectioned and hybridized with RNA-FISH probes to VDV-like capsid RNA to show the localization of DWV/VDV-like viruses within the tissue.

2.7.1 Stellaris-RNA FISH

Custom Stellaris® RNA FISH Probes were designed against the published capsid sequence for VDV (GenBank: AY251269.2) by utilizing the Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner. The sectioned honey bee tissue was hybridized with the VDV Stellaris FISH Probe set labelled with CAL Fluor® Red 590 (Biosearch Technologies, Inc.), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols.

2.7.2 Probe Design

The Stellaris probes cannot distinguish between sequences with high levels of homology, so *in silico* testing was carried out to determine the best sequence upon which to base the probe design in order to give either a). the ability to accurately distinguish between VDV-like and DWV-like sequences or - if the technology was found to be unable to distinguish between the two - b). a probe set which will pick up all variants.

Sequence homology in the capsid region of the two most distantly related DWV-like sequences on Genbank (determined by clustal alignment) is 84%. These capsid sequences are not distinct enough to be differentiated by Stellaris probes, according to the manufacturer's literature, so I now needed to determine whether a probe set designed to detect one capsid sequence would successfully detect all DWV-like viruses.

A probe set was designed to anneal to the VDV-like capsid sequences, as this is the most prevalent in local bees and the predominant strain found in the virus prep used for all experimental infection assays. For the designed probe set, a BLAST search was conducted. As expected, the probes were all a 100% annealing match to the VDV-like capsid sequence for which they were designed (accession number KJ437447.1), when aligned to a DWV-like capsid (accession number [KX580899.1](#)) 80% (40/50 probes) were a 100% identity match for the template sequence, with the remaining 20% (10/50) having just one mis matched base. It is recommended by the manufacturer that at least 4 mismatches are required to prevent a probe binding to a non-target sequence, and that a probe set of 25+ is sufficient to give full on target annealing. Change this: Therefore it was concluded that the probe set designed would be likely to detect both VDV-like and DWV-like viral capsid sequences.

2.7.3 Processing of experimentally infected honey bees for RNA-FISH

All tissue fixed in Kahle's solution and then stored at 4°C until use.

Mix:

17 ml Ethanol EtOH,

6 ml Formalin H₂C(OH)₂,

28 ml RNase free water ddH₂O,

2 ml Glacial acetic acid CH₃CO₂H.

Initial dissection

Individual bees/pupae were divided into 3 sections – head, thorax and abdomen and placed into a histology cassette for dehydration. Larvae were left whole up until the 4th instar, with older larvae and propupae being cut into two pieces (head and body).

Dehydration of tissue

Slow dehydration for long periods on ethanol series ranging from 15% to 100%, diluted in DEPC treated distilled water (ddH₂O). Cold ethanol solutions with gentle shaking (at 4°C) of samples during all the time of dehydration process, with 2 hours in each ethanol solution. The dehydration and embedding protocols are described in (Silva-Zacarin, *et al.*, 2012).

The series of ethanol solutions were as follows:

15%, 30%, 50%, 70%, 80% (overnight at 4°C), 85%, 90%, 100%, 100% (overnight at 4°C).

Paraffin embedding method

After fixation and dehydration, samples were gently transferred into small glass containers. HistoClear II (National Diagnostics (Luchtel, *et al.*, 1998)) was used for clearing the tissue in place of the Xylene recommended by (Silva-Zacarin, *et al.*, 2012) as it is nontoxic. Samples were placed into a solution containing 50% HistoClear II:50% ethanol for 1 hour, with occasional shaking at room temperature.

This solution was subsequently replaced by a 75% HistoClear II:25% ethanol solution and incubated for 1 hour.

This solution was then replaced with 100% HistoClear II three times for at least 1 hour each at room temperature, in order to remove completely the ethanol from all tissues.

Paraplast X-TRA (Sigma-Aldrich) chips were added gradually in the glass containers containing the samples and finally placed in a heat block at 60°C for 2 hours. Molten Paraplast X-TRA was then replaced three times for at least 8 hours each time to obtain optimal infiltration of paraffin into the whole larvae. Note: samples can stay few days in Paraplast before polymerization.

The embedding moulds containing molten Paraplast X-TRA were placed on a hot plate (50°C) and the larvae were oriented in these moulds with warmed tweezers. After Paraplast X-TRA polymerization, paraffin blocks were stored at 4°C until use.

Sectioning embedded material

The paraffin-embedded tissues were attached to the back of the histology cassette and the edges shaped with a razor blade to form a pyramid. Ribbons of 8 µm thickness were generated using a microtome and placed on ddH₂O coated slides (Adhesion slides, SuperFrost® Plus, VWR) with the ribbon shiny side toward the water surface. Excess water was removed and slides were incubated overnight at 35°C to promote an optimal tissue adhesion to the slide. Slides were then stored in dry boxes at 4°C until use.

Deparaffinisation of formalin-fixed paraffin embedded tissue sections

Briefly, the Paraplast X-TRA embedding medium was removed from the slides through immersion in Histoclear II. The slides were then immersed in 100% EtOH for 20 mins, 95% EtOH for 10 mins and then 70% EtOH for at least 1 hour to re-hydrate and permeabilise the tissue sections. Immediately prior to hybridization, slides were removed from the ethanol and placed into RNase free PBS (5 min), followed by 20 mins in PBS with proteinase K (10 µg/ml) at 37°C. Finally, slides were equilibrated in wash buffer (see below) for 5 mins.

Hybridisation of tissue

The Stellaris-FISH probe is supplied as a dried stock. Initial dilution was in TE buffer to 12.5 μ M concentration, with the diluted probe solution being stored at -20°C. This was then further diluted to a working solution of 2.5 μ M in TE buffer (this concentration was selected after initial testing of the probe set at different dilutions – 1:10, 1:2.5, 1:5, 1:10 – as per manufacturers instructions). 2 μ l of the ‘working solution’ of the probe was added to 100 μ l of hybridization buffer (see below) per slide, this mix was pipetted over the slides and then covered with a clean cover slip and incubated over night at 37°C in a humidified chamber. Finally, the slides were immersed in wash buffer for 30 min, dried and mounted in ProLong Diamond AntiFade Mountant with DAPI (Invitrogen).

Hybridization Buffer:

Mix:

1 g Dextran Sulfate
1 ml 20x Saline-Sodium Citrate (SSC) buffer
1 ml deionized formamide
+ Nuclease free water to 10 ml final volume.

Wash Buffer:

Mix:

5 ml 20x SSC buffer
5 ml deionized formamide
+ Nuclease free water to 50 ml final volume.

2.8 Confocal Microscopy

All imaging took place using the Zeiss 880 confocal microscope, (Imaging suite, University of Warwick). Samples that were known to have either high or low levels of

DWV were prepared and used to set the parameters for laser illumination levels and objectives. For all images, tile scans of 5x5 images with the 25x objective were recorded, to allow for a 1500 µm square section of tissue to be analysed per image. Filters were set for one channel (to detect DAPI) at 410 nm to 566 nm with the 651 nm excitation laser at 2%, and a second channel (to detect the Stellaris probe, labelled CAL Fluor® Red 590, hybridised to DWV) between 585nm and 733nm, excitation from laser at 405 nm, 3.5%. Gain and offset settings were optimised for the control slides and remained unchanged for all images.

Slides had been prepared with the insects pre-dissected – with larvae and propupae divided into ‘head’ and ‘body’ segments and pupae and adults divided into ‘head’, ‘thorax’ and ‘abdomen’. Identification of tissue types within segments was carried out in reference to (Stell, 2012). For each time point, three individual insects were examined per treatment, with at least three images of each body segment being quantified per individual. To avoid the introduction of researcher bias into the selection of imaging sites, all slides were examined ‘blind’ with the treatment type obscured until after the images had been processed and quantified.

2.8.1. Image processing

All images were processed using Fiji (Schindelin, *et al.*, 2012), an image processing package of the open platform, public domain, Java based imaging program developed by the National Institutes of Health (Schindelin, *et al.*, 2015). To quantify the levels of fluorescence for both DWV (CAL Fluor® Red 590, Stellaris Probe) and for DAPI (the nuclear stain 4', 6-diamidino-2-phenylindole, fluorescence at 461 nm when bound to double stranded DNA within cells (Kubista, *et al.*, 1987)) images were converted to RGB colour and then quantified using the Colour Pixel Counter plugin (author Ben Pichette). The number of red pixels per image (DWV) was normalised to the number of blue pixels (DAPI) to control for difference in tissue density.

2.9 Statistics

All data analyses were performed using SPSS version 24 (SPSS, Inc., Chicago IL).

Where both deltaCt and calculated viral copy numbers are shown, the viral copy numbers are used for statistical analysis.

Statistical tests were selected according to the data set, further details are given in the results sections of chapters 3, 4 and 5.

Chapter 3. Principals of DWV Infection. Investigation of how DWV transmission route influences virus accumulation and localization.

3.1. Introduction

In this chapter, a range of experiments are described that simulate physical transmission of deformed wing virus into adult, larval and pupal honeybees. Both oral transmission to larvae, and *Varroa* mediated transmission to pupae and adults were studied. The latter was done by injection of a DWV preparation into bees maintained under controlled environment conditions in the laboratory.

DWV was first found and characterised in the UK in the 1980's, before the introduction of the *Varroa* mite (Bailey, *et al.*, 1981) when examination of deformed (symptomatic) bees revealed high titres of an icosahedral single stranded RNA virus. However, DWV was not seen as a major issue by beekeepers or researchers and symptomatic outbreaks were very rare (Allen and Ball, 1996). DWV was rarely present at levels high enough to be detectable by the techniques of the time. The honey bee research community is currently undecided as to the prevalence of DWV in honey bee colonies prior to the introduction of *Varroa* (de Miranda and Genersch, 2010). Sensitive molecular techniques including qPCR and Illumina sequencing have revealed that low level, asymptomatic DWV infection can be detected in colonies from *Varroa* naive areas, such as those on Colonsay island (an island in the Inner Hebrides of Scotland, currently one of the few remaining *Varroa* free areas of Europe) and those in Northern Scandinavia, beyond the expansion front of *Varroa* (Yue and Genersch, 2005; de Miranda and Fries, 2008; Ryabov, *et al.*, 2014b). However, samples from Australia (the last large land mass to remain free of the parasite) have been shown to be DWV free (Roberts, *et al.*, 2017). Whether the virus could have been introduced by beekeeping practices into these European samples, or whether it was widely present within wild bee populations in the

area, forming a persistent, asymptomatic infection, the fact remains that in the absence of *Varroa* the virus is capable of establishing a covert, asymptomatic infection.

DWV replication has also been detected in bumble bees (both *Bombus pascuorum* and *Bombus terrestris*) and in a number of other arthropods that are associated with honey bee hives, including the European earwig (*Forficula auricularia*) and the German cockroach (*Blattella germanica*) (Genersch, *et al.*, 2006; Levitt, *et al.*, 2013). These species are not vulnerable to *Varroa* parasitism, so they are thought to become infected through direct association with honey bee colonies, and possible ingestion of contaminated food or pollen.

Upon the introduction of *Varroa* to the UK, the prevalence of apparent, symptomatic infections by certain viruses (DWV, cloudy wing virus (CWV) and slow paralysis virus (SPV)) increased markedly (Martin, *et al.*, 2010; Martin, *et al.*, 2013). A comprehensive sampling and monitoring project, conducted by the UK National Bee Unit has given a reliable picture of the ‘post *Varroa*’ viral landscape in honey bees in England and Wales. As anticipated, weaker colonies were found to be more frequently associated with higher numbers of pathogens. One key finding of the survey was that, of all of the pathogens tested for, DWV was the only one that could be reliably used as an indicator of colony strength, with colonies that had high levels of DWV being consistently more likely to have fewer combs of brood or bees (Budge, *et al.*, 2015). A similar project in Germany found that high levels of DWV and acute bee paralysis virus (ABPV) in the autumn, along with a heavy *Varroa* mite load, were significantly related to overwinter colony losses (Genersch, *et al.*, 2010). DWV has also been implicated in the rise in colony losses in Greece (Bacandritsos, *et al.*, 2010) and Switzerland (Dainat, *et al.*, 2012b). However the picture is less clear in the USA, where research has focused on ‘Colony Collapse Disorder’ (CCD), with DWV being amongst the set of pathogens that were found to be moderately higher in colonies that had suffered from CCD in one study (Cornman, *et al.*, 2012), but not amongst the most significant predictors reported by (vanEngelsdorp, *et al.*, 2009).

In the absence of *Varroa*, transmission of honey bee viruses including DWV occurs through two separate pathways: either vertical or horizontal (Fig 3.1). Vertical transmission is the transfer of an infection between generations, from parent to offspring. Vertical transmission of DWV has been experimentally shown between drones and queens during mating and between queens and eggs (Yue, *et al.*, 2007; de Miranda and Fries, 2008; de Miranda and Genersch, 2010). This DWV transmission route results in persistent, covert, asymptomatic infections and is in line with a general hypothesis that vertical pathogen transmission leads to the selection of less virulent strains, that allow the infected individual to survive to adulthood (Ewald, 1983; Fries and Camazine, 2001). Horizontal transmission occurs between members of the same generation (in honey bees, this occurs between members of the worker caste and drones within a colony (Chen, *et al.*, 2006a)) (Fig 3.1). Horizontal transmission of DWV has been shown to occur via food given to larvae and transferred between adults and results in covert infections (Yue and Genersch, 2005; Gisder, *et al.*, 2009). DWV has also been shown to be present in the midgut of asymptomatic individuals (Fievet, *et al.*, 2006) and in the faeces of adult honey bees (Chen, *et al.*, 2006b).

The vertical and horizontal routes of infection offer some explanation as to how DWV is maintained in honey bee populations consistently and at low levels in the absence of *Varroa* without undergoing the periodic, host density-dependent epizootics that are common in other viral pathogens. However, the introduction of *Varroa* mites to *A. mellifera* dramatically changed the population dynamics of DWV. Two hypotheses have been put forward to explain the increase in the prevalence of symptomatic infections by DWV associated with the introduction of *Varroa*. The first hypothesis is that *Varroa* parasitism of developing pupae has an immunosuppressive effect, which allows viral infections vectored by the mite to progress unchecked in naïve honeybee hosts. Some other species of ectoparasites have been shown to suppress the immune response of the host and it has been proposed that this is an adaptation that facilitates parasite reproduction or feeding and also benefits the transmission of pathogens (Zhao, *et al.*, 2009; Herniou, *et al.*, 2013). For example, the tick *Ixodes scapularis* is a vector of pathogens including Lyme disease in the Eastern United States. In order to secure a

blood meal from their mammalian hosts, the ticks must feed for several days. They have been shown to counter the immune responses of their hosts to facilitate this feeding, and this is achieved through salivary proteins which inhibit T-cells, B-cells, the complement systems and the coagulation system (Schuijt, *et al.*, 2011). It has also been shown that the host response to *Borrelia burgdorferi* (the spirochete that causes Lyme disease) differs depending on how the disease is transmitted – by the tick or experimentally via a needle. This is due to both the tick suppression of the host immune response, and due to the modification of the Osp-A surface protein on the spirochete during tick feeding. The presence Osp-A is necessary to induce the host development of antibodies to the spirochete (Schwan, *et al.*, 1995; Wikel, 1999).

In some examples, mutualistic symbiosis between vectors and the pathogens they transmit is highly co-evolved, for example in the case of parasitoid brachonid and ichneumonid wasps, where polydnviruses are integrated into the parasitoid genome, allowing the wasps to produce and use functional modified virus particles containing fragmented dsDNA genomes which encode virulence genes (Zhao, *et al.*, 2009). These virions are utilised by the parasitoids to evade or suppress the immune systems of their lepidopteran hosts, injecting them alongside their eggs to prevent encapsulation by haemocytes (Federici and Bigot, 2003; Herniou, *et al.*, 2013). In contrast, the interaction between *Varroa*, DWV and *A. mellifera* is relatively recent in evolutionary terms (Locke *et al.* 2012), and hence it is premature to speculate as to whether there is a mutualistic interaction in *A. mellifera* hosts. There is some evidence from laboratory experiments that the saliva injected by *Varroa* into honey bees has an immunosuppressive effect on the host, explaining the increased virulence of DWV when it is associated with *Varroa* (Yang and Cox-Foster, 2005; Rosenkranz, *et al.*, 2006; Dainat, *et al.*, 2012a; Nazzi, *et al.*, 2012; Francis, *et al.*, 2013) although this finding has been disputed by Kuster *et al.* (2014), who found no evidence for immunosuppression in *Varroa* parasitized bees (Kuster, *et al.*, 2014), while an *in vitro* study of the effects of *Varroa* infestation on developing worker bees showed increased expression of several immune genes (Gregorc, *et al.*, 2012). To date, there has been little work published that separates the effect on honey bees of *Varroa* feeding on its own from the impact of

Varroa-mediated DWV infection due to the ubiquitous association of DWV with *Varroa* mites. The *in vitro* collection of *Varroa* saliva has allowed a preliminary biochemical characterization of the secretions, with 15 distinct proteins present in the saliva. The *Varroa* saliva was also shown to impair the function of *Lacnobia oleracea* haemocytes in culture, indicating that these salivary proteins may facilitate the suppression of haemocyte-mediated wound healing (Richards, *et al.*, 2011).

A second explanation for the increased virulence of DWV when associated with *Varroa* mites, is that the feeding mite has opened up a new route of transmission for DWV by injecting it directly into the honey bee haemocoel, allowing the virus access to a wider range of tissues than it would otherwise have through infection *per os*, and bypassing co-evolved host defences.

Alternatively, there could be some filtering or preferential selection of certain DWV sequences or strains when transmission is via *Varroa* mites (Ryabov, *et al.*, 2014; Gisder, *et al.*, 2018), and these selected strains could in turn be more pathogenic when injected into the honey bee during *Varroa* feeding. There is more focus on the selection of virulent strains in Chapter 4 of this work.

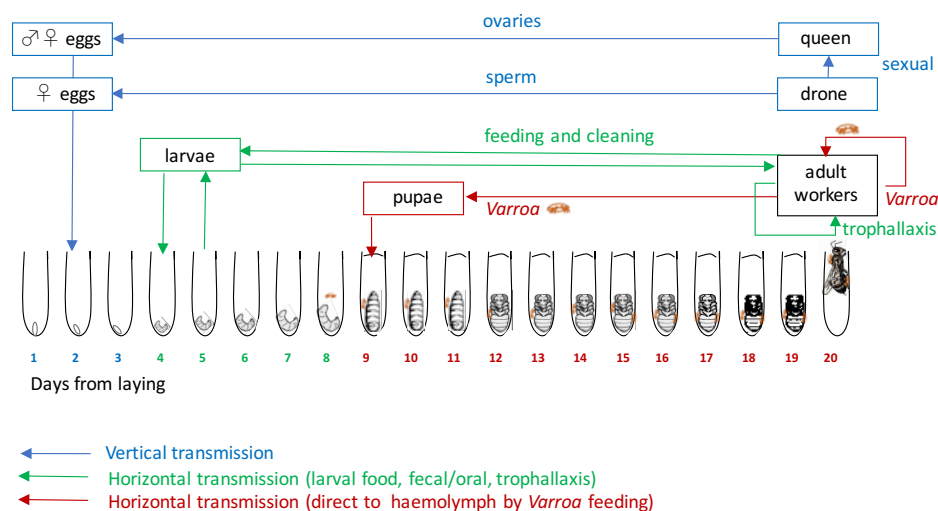


Figure 3.1 Defining the different possible transmission routes for honey bee viruses. Adapted from de Miranda *et al.* (2011) and COLOSS BEEBOOK Vol 1.

It has been shown that symptomatic, *Varroa* exposed individuals have DWV present in total RNA extracted from their heads, as do individuals experimentally injected with DWV to mimic *Varroa* feeding (Yue and Genersch, 2005; Iqbal and Mueller, 2007), this infection of the brain is absent in vertically or orally exposed individuals. Experimental injection of DWV into bees has been shown to cause subtle learning deficits in adult bees and ‘classical’ DWV symptoms (deformed wings, stunted abdomen) in individuals that were injected as pupae (Moeckel, *et al.*, 2011).

It is clear from the literature that the route through which an individual honey bee is exposed to DWV is crucial in determining if a symptomatic infection will develop. There is also persuasive evidence that different tissue types are infected in asymptomatic and symptomatic infections. However, there is little detailed knowledge on the pattern of tissue infection following oral or *Varroa* mediated infection, or on the time course of infection following controlled dose experimental infection.

In this chapter, a series of experiments were conducted using larval feeding or injection techniques to reveal more about how the mode of transmission affects the pathogenicity of the virus and how this is linked to virus localisation and pupal development. The viral outcomes (determined by qPCR, calculating DWV copy number per individual) as a result of oral exposure (larval) or injection (pupae, adults) of DWV virus prep were established. This ‘dose response’ information was followed by time course experiments, where a group of individuals were administered a single dose of virus and then sequentially sampled in order to reveal more about the time course of infection. A subset of individuals was collected from each sampling point and fixed to allow visualization of DWV localisation during infection using RNA-FISH and confocal microscopy.

3.2 Methods

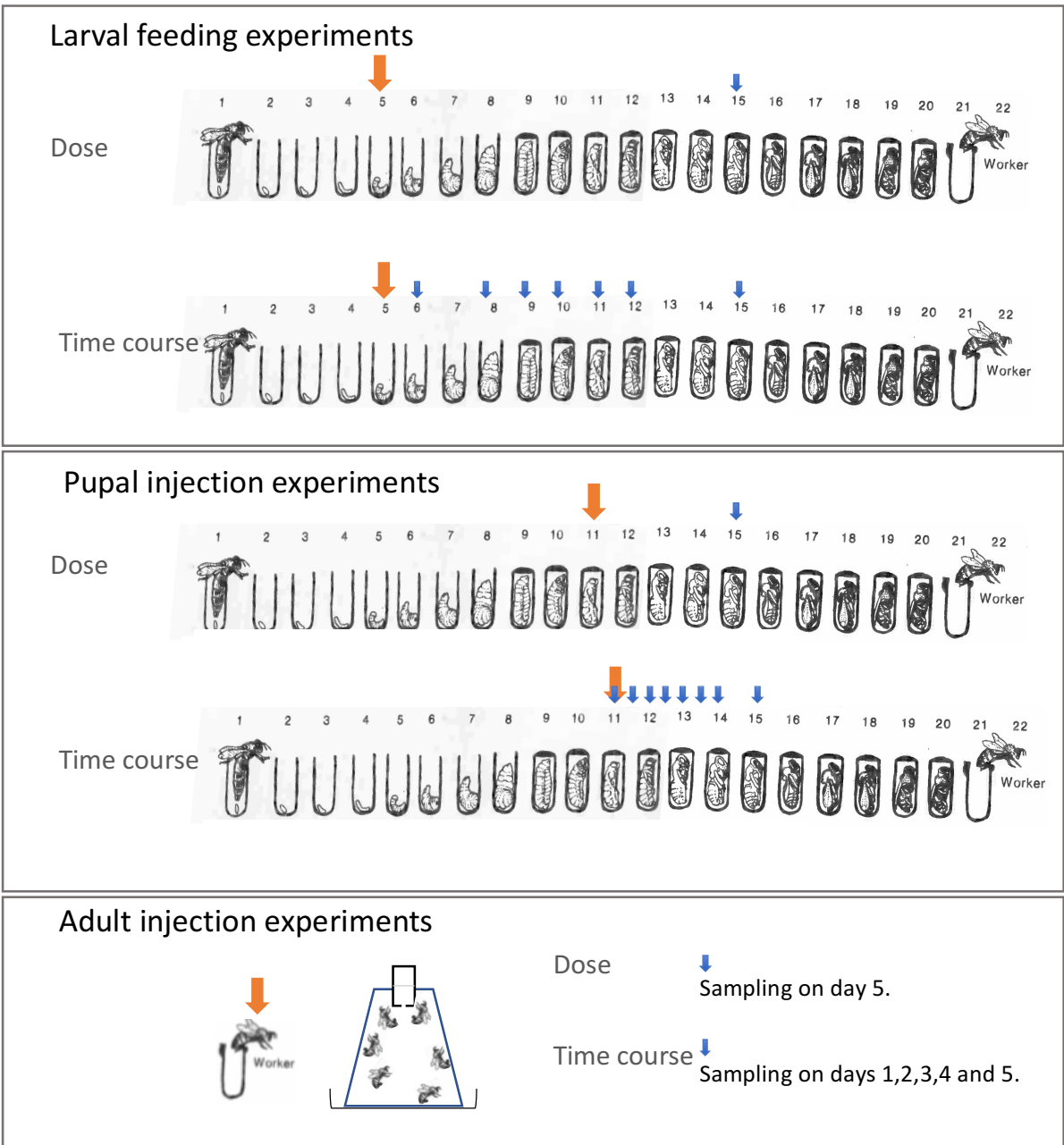


Figure 3.2 Sampling times for feeding and injection experiments. Time of exposure to DWV shown by orange arrows, sampling points shown by blue arrows. Worker development figure adapted from (Winston, 1987; Fahrbach, et al., 2012)

Techniques for larval rearing, pupal injection and adult injection experiments were described in Chapter 2 of this thesis, sections 2.3, 2.4. and 2.5, and the DWV virus preparation method is given in section 2.2. The experiments were conducted as follows:

3.2.1 Characterisation of DWV virus preparation

All experiments in this chapter were conducted using a virus preparation (VP) from symptomatic honey bees (Wellesbourne 2015 VP). Before it could be used to infect bees, PCR and qPCR tests were carried out on the VP cDNA to give an indication of the strain of DWV present and check that no other honey bee viruses were present.

Virus prep was ‘cleaned up’ using the GeneJet RNA Purification kit (Thermo Scientific. RNA clean up protocol) and then used as a template for reverse transcription, to give cDNA. This cDNA was then used as a template for PCR reactions with primers to amplify Sacbrood Virus (SBV), Acute Bee Paralysis Virus (ABPV), Israeli Acute Bee Paralysis Virus (IABPV) and Chronic Bee Paralysis Virus (CBPV) and Deformed Wing Virus (DWV). A qPCR run was carried out with DWV strain specific primers following the protocol in Chapter 2 (Section 2.6.2.). A full list of primers can be found in Appendix 1 of this work.

3.2.2 - Quantification of DWV in honey bee pupae following inoculation via larval food

This involved giving a single dose of DWV VP to young honey bee larvae (5 d old from egg laying.) This experiment recreated direct horizontal transmission of DWV within a colony, specifically the transfer of the virus from nurse bees to developing larvae during feeding, the primary route via which DWV is thought to persist in a colony in the absence of *Varroa* mites. Dose ranges and sampling times were established during preliminary experiments.

3.2.2.1. Dose Response - Quantification of DWV in honey bee pupae following inoculation via larval food

The basic *in vitro* larval maintenance protocol is given in Chapter 2 (2.3.1). This protocol was modified by mixing a dose of DWV into the larval food given on day one of the assay. The doses given were as follows: 5.56×10^{13} DWV copies/larvae, 5.56×10^{11} DWV copies/larvae, 5.56×10^{10} DWV copies/larvae, 5.56×10^9 ng DWV copies/larvae, 5.56×10^7 ng DWV copies/larvae and 5.56×10^5 DWV copies/larvae, plus a control dose containing no DWV. Sampling took place at the purple eye stage of pupal development, 10 days after DWV exposure. The experiment was repeated on two separate dates, and in each replicate 12 individuals treated at each dose. Sampling, RNA extraction and qPCR analysis were carried out as in Chapter 2, sections 2.5 and 2.6.2.

3.2.2.2. Time to Response - Quantification of DWV in honey bee pupae following inoculation via larval food.

The basic *in vitro* larval maintenance protocol is given in Chapter 2 (2.3.1). This protocol was modified by mixing a dose of DWV into the larval food given on day one of the assay. The doses given were as follows: 5.56×10^{13} DWV copies/larvae or a control dose (DWV free food). Sampling took place at set times throughout the experiment, at 24 h, 72 h, 96 h, 144 h, 168 h and 240 h post DWV exposure. The experiment was repeated on two separate dates, and in each replicate 40 individuals were treated at each dose. Sampling, RNA extraction and qPCR analysis were carried out as in Chapter 2, sections 2.5 and 2.6.2.

3.2.3. Quantification of DWV in honey bee pupae following inoculation via injection

These experiments involved using a Hamilton syringe to administer a single dose of DWV VP to honey bee pupae at the ‘white eye’ stage of development to mimic the

direct transfer of the virus to honey bee haemolymph during *Varroa* feeding. As described in Chapter 1, *Varroa* mites reproduce within capped cells in honey bee colonies, feeding upon developing pupae. *Varroa* feeding has actually been documented to start within 5 hours of the cell being capped, whilst the inhabitant bee is still at the prepupal stage of development (Kanbar and Engels, 2003; Rosenkranz, *et al.*, 2006). However, preliminary experiments established that it was not possible to successfully inject prepupae due to the pliability of the cuticle and lack of internal structure, which cause leaking of haemolymph and death following injection. Injection immediately following pupation, at the white eye stage of development, was found to be more reliable. This is the stage of development at which the mother mite would make the final feeding perforation in the second abdominal segment - immediately prior to sclerotisation of the pupal cuticle – which is then used by the mother mite and her offspring throughout the pupal stage (Kanbar and Engels, 2003). Dose ranges and sampling times were established during preliminary experiments.

3.2.3.1 - Dose Response - Quantification of DWV in honey bee pupae following inoculation via injection.

White eye pupae, day 12 to 13 from laying (4 days post capping) were injected with a single dose of DWV VP, doses ranged from 1.85×10^2 viral copies per individual, to 1.85×10^8 viral copies per individual. A single injection was given into the pupal haemocoel on day one of the experiment, as described in Chapter 2, section 2.4.1. Sampling took place 4 days post injection, at the purple eye stage of development. The experiment was repeated on two separate dates, with 6 individual bees per treatment in each replicate. Sampling, RNA extraction and qPCR analysis were carried out as in Chapter 2, sections 2.5 and 2.6.2.

3.3.3.2. Establishing that ‘high DWV’ individuals go on to become symptomatic adults

White eye pupae, day 12 to 13 from laying (4 days post capping) were injected with a single dose of DWV VP, 1.85×10^6 viral copies (n=50), or with a control injection (n=25). A single injection was given into the pupal haemocoel on day one of the experiment, as described in Chapter 2, section 2.4.1. The injected pupae were left to develop to adulthood in the incubator (12 days) and then scored as having either normal or deformed wings, and normal or stunted abdomen.

3.2.3.3. Time to Response - Quantification of DWV in honey bee pupae following inoculation via injection

White eye pupae, day 12 to 13 from laying (4 days post capping) were injected with a single dose of DWV VP, 1.85×10^6 viral copies, or with a control injection. A single injection was given into the pupal haemocoel on day one of the experiment, as described in Chapter 2, section 2.4.1. Sampling took place at planned time points throughout the experiment, at 0 h, 10 h, 23 h, 33 h, 48 h, 58 h, 72 h, 80 h, 96 h and 105 h post DWV exposure. The experiment was repeated on two separate dates, with 60 individual bees per treatment in each replicate. Sampling, RNA extraction and qPCR analysis were carried out as in Chapter 2, sections 2.5 and 2.6.2.

3.2.4. Quantification of DWV in honey bee adults following inoculation via injection

These experiments involved using a Hamilton syringe to administer a single dose of DWV VP to newly emerged worker bees to reveal more about the effect of phoretic *Varroa* mites feeding on adult bees (Genersch, *et al.*, 2005; Piou, *et al.*, 2016). Newly emerged individuals were used despite *Varroa* mites documented preference for nurse bees over newly emerged individuals or foragers (Xie, *et al.*, 2016). This was because it was necessary to watch the bees emerge from the frame to ensure that they were *Varroa* free prior to injection. An additional advantage is that newly emerged bees are unable to

sting, meaning that they could be injected without immobilising via chilling or CO₂ anaesthesia, which have been shown to cause premature aging and reduced lifespan of worker honey bees (Human, *et al.*, 2013). Dose ranges and sampling times were established during preliminary experiments.

3.2.4.1 - Dose Response - Quantification of DWV in honey bee adults following inoculation via injection

Newly emerged adult worker bees were injected with a single dose of DWV VP, doses ranged from 1.85×10^2 viral copies per individual, to 1.85×10^8 viral copies per individual. A single injection was given into the abdomen day one of the experiment, as described in Chapter 2, section 2.5. Sampling took place 4 days post injection. The experiment was repeated on two separate dates, with 6 individual bees per treatment in each replicate. Sampling, RNA extraction and qPCR analysis were carried out as in Chapter 2, sections 2.5 and 2.6.2.

3.2.4.2. Time to Response - Quantification of DWV in honey bee adults following inoculation via injection

Newly emerged adult worker bees were injected with a single dose of DWV VP, 1.85×10^6 viral copies or with a control injection. A single injection was given into the pupal haemocoel on day one of the experiment, as described in Chapter 2, section 2.5.

Sampling took place regularly throughout the experiment, at 0 h, 10 h, 23 h, 33 h, 48 h, 58 h, 72 h, 80 h, 96 h and 105 h post DWV exposure. The experiment was repeated on two separate dates, with 60 individual bees per treatment in each replicate. Sampling, RNA extraction and qPCR analysis were carried out as in Chapter 2, sections 2.5 and 2.6.2.

3.2.5 Localisation of DWV in honey bees using Stellaris probes (RNA-FISH) and confocal microscopy

Samples for RNA-FISH were produced by conducting exact repeats of the ‘Time to Response’ experiments detailed above in sections **3.2.2.2.** and **3.2.3.2.** to allow visualisation of the time course of infection in honey bees exposed to DWV via food or injection. Samples were fixed and processed as is described in Chapter 2, section **2.8.3.** All imaging took place using the Zeiss 880 confocal microscope, as in Chapter 2, section **2.9.**

3.3 Results

3.3.1 Characterisation of Virus Preparation used for Horizontal Transmission of DWV Experiments – Oral exposure and direct injection

All experiments in this chapter were conducted using a virus preparation (VP) from symptomatic honey bees (Wellesbourne 2015 VP). Before it could be used to infect bees, PCR and qPCR tests were carried out on the VP cDNA to give an indication of the strain of DWV present and check that no other honey bee viruses were present. PCR results showed that the VP was positive for DWV, but free of Sacbrood (SBV), Acute Bee Paralysis Virus (ABPV), Israeli Acute Bee Paralysis Virus (IABPV) and Chronic Bee Paralysis Virus (CBPV) (Figure 3.3, A).

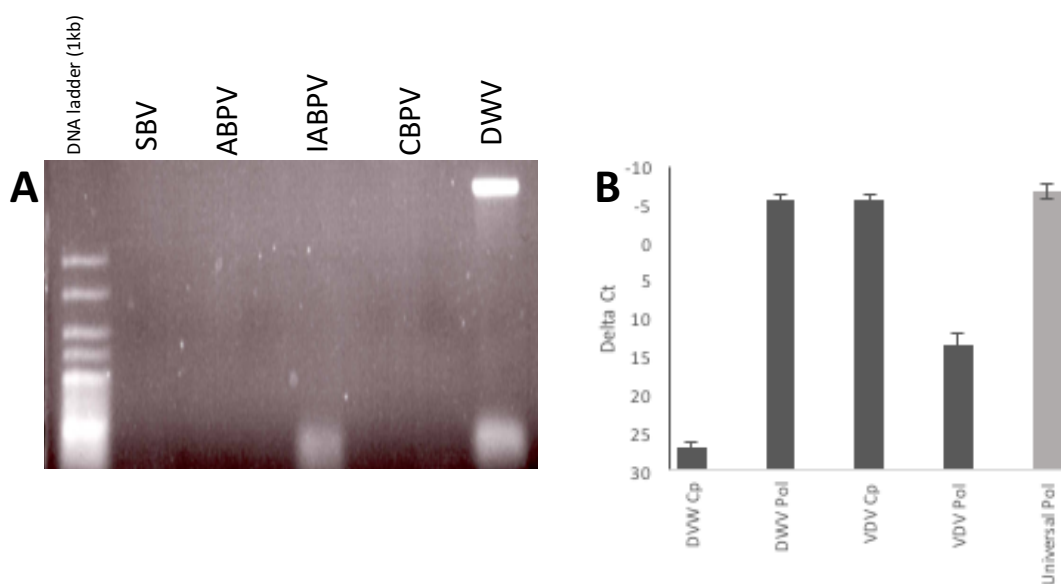


Figure 3.3. PCR analysis of virus prep cDNA. Prep is positive for DWV but Sacbrood virus (SBV), Acute Bee Paralysis Virus (ABPV), Israeli Acute Bee Paralysis Virus (IABPV) and Chronic Bee Paralysis Virus (CBPV) are absent. 500bp ladder. B – Strain specific qPCR of VP cDNA, indicating that both recombinant VDV/DWV type virus and full length VDV-1 type virus are present in VP2015. Cp bars represent viral capsid sequences, Pol bars represent polymerase sequences.

The VP cDNA was then examined using DWV strain specific qPCR to give an indication of the predominant strain of DWV or VDV in the prep. This gives a picture of the relative abundance of DWV-like or VDV-1-like structural (Cp) and non-structural (Pol) sequences within the sample. The results indicate that there are high levels of DWV-like polymerase and VDV-1 like capsid sequences present in the VP, with VDV-1 like polymerase sequences also represented, although at lower levels. This result is in line with our previous findings that a virulent DWV/VDV-1 recombinant strain of the virus dominates the viral population in our local, *Varroa* exposed, honey bee colonies (Ryabov, *et al.*, 2014b).

3.3.2 Larval feeding experiments

3.3.2.1 Dose Response - Quantification of DWV in honey bee pupae following inoculation via larval food

In order to learn more about how DWV infects honey bees and how this infection process is influenced by the mode of transmission of the virus, dose response curves were first established from the virus preparation (Wellesbourne 2015 VP) for the two routes of transmission – oral or direct injection. It has been established in the literature that symptomatic individuals typically have a viral load of over 10^9 copies of DWV per individual, and that the infection is typically bimodal, with a clear distinction between ‘infected’ individuals with viral loads of around 10^9 or above, and asymptomatic individuals with significantly lower viral loads (Zioni, *et al.*, 2011; Ryabov, *et al.*, 2014a).

Oral exposure of honey bee larvae to DWV VP, via a single dose at 5 days from laying in larval feeding experiments, resulted in a bimodal distribution in observed DWV

levels in the experimental insects, when sampled at purple eye pupal stage (12 days post virus exposure). Individuals given a ‘low’ dose of the VP (5.56×10^5 DWV copies/larvae to 5.56×10^{11} DWV copies/larvae) had DWV levels indistinguishable from the control bees. However, larvae given a very high dose of DWV (5.56×10^{13} DWV copies/larvae) went on to have elevated DWV levels, in line with those observed in symptomatic adult honey bees (Figure 3.4). There was a statistically significant difference between the treatment groups as determined by one-way ANOVA ($F(4,20) = 11.271, p < 0.05$). A Tukey post hoc test revealed that only the 5.56×10^{13} DWV copies/larvae treatment was significantly different from the other treatments and the control group.

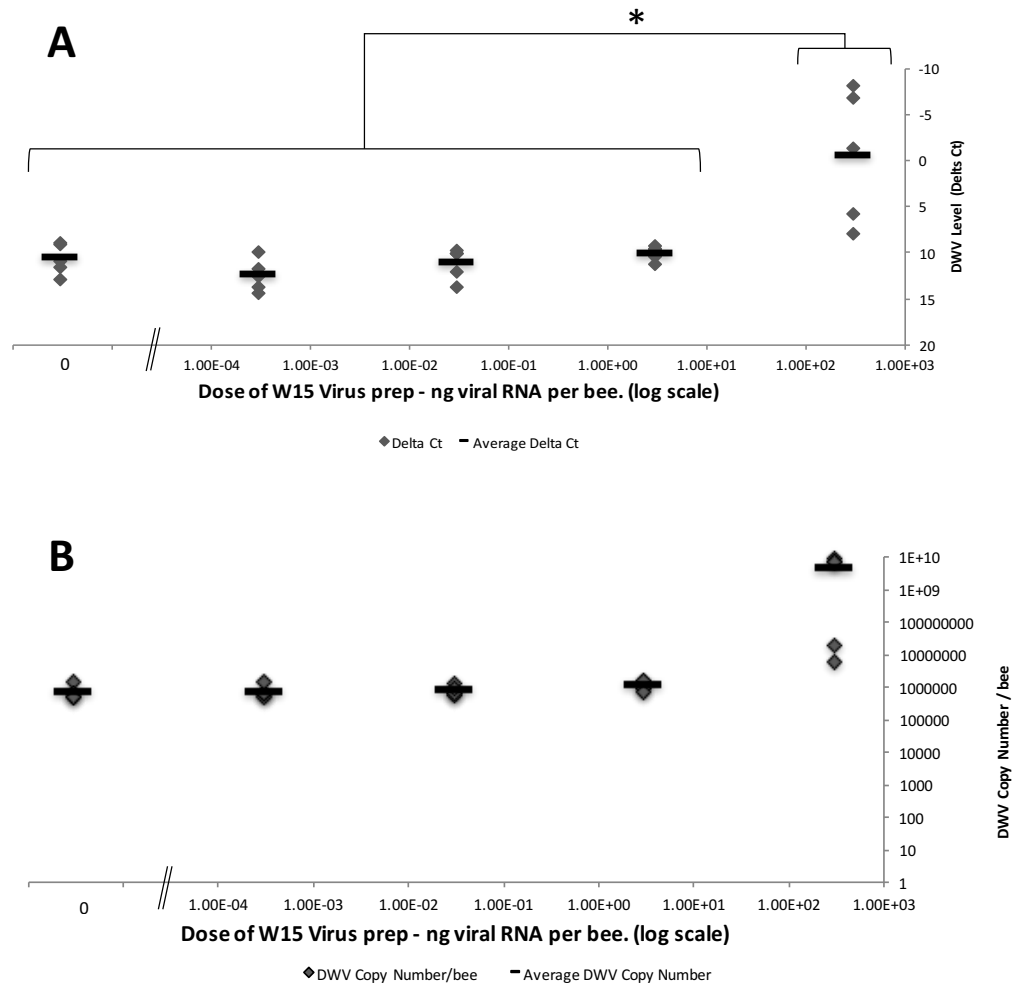


Figure 3.4. Dose response curves for oral exposure of DWV. Doses given to larvae from a single colony at 5 days post laying. Sampling at ‘purple eye’ larval stage, 10 days post virus exposure. A. Delta Ct for Universal DWV primers, normalised to Actin. B. DWV copy number. * denotes treatment groups shown to have a statistically significant viral outcome.

3.3.2.2. Time to Response - Quantification of DWV in honey bee pupae following inoculation via larval food

As described above, honey bee larvae are able to be exposed to relatively high doses of DWV without succumbing to infection. In order to have any ‘high virus’ outcome individuals upon sampling at the purple eye stage of development, a dose of 5.56×10^{13}

DWV copies/larvae must be given early in larval development. Lower doses or later exposure resulted in individuals with low levels of DWV infection, similar to those found in control (non DWV exposed) bees.

In order to learn more about how this high dose of DWV is able to overcome the host defences and cause symptomatic infections, experiments were conducted in which larvae were given a single, infectious dose of DWV (5.56×10^{13} DWV copies/larvae) and then samples were collected at regular time points throughout the larval stage and pupation. The results of this experiment are shown in figure 3.5. DWV levels were similar for control and treatment groups for the first 4 days of sampling, however at 6 days post viral inoculation the samples from the virus exposed group had higher levels of DWV than those fed a DWV free diet. A two-way ANOVA was conducted that examined the effect of treatment (DWV or Control) and time on viral outcome. There was a statistically significant interaction between the effects treatment and sampling time on the level of DWV at sampling, $F(5, 63) = 14.215, p < 0.005$.

Post hoc analysis (Tukey HSD) reveals that there is no difference between the DWV levels in the control and in the treatment groups until 6 days post infection. This is the point at which the propupae is undergoing metamorphosis prior to the fifth moult where the pupal form is revealed (Stell, 2012). During honey bee development, the first physiological change to the larvae following its final meal and the capping of the cell for pupation, is the opening of the midgut, hindgut and malpighian tubules to allow waste to pass from the larval stomach and be evacuated into the bottom of the cell. The empty intestines then undergo extensive remodelling during pupation. It is possible that, if DWV is present during this process it may be able to access tissues that had previously been protected by the physical barrier of the larval midgut walls, leading to the elevated DWV levels that were seen in pupae 6 days post infection.

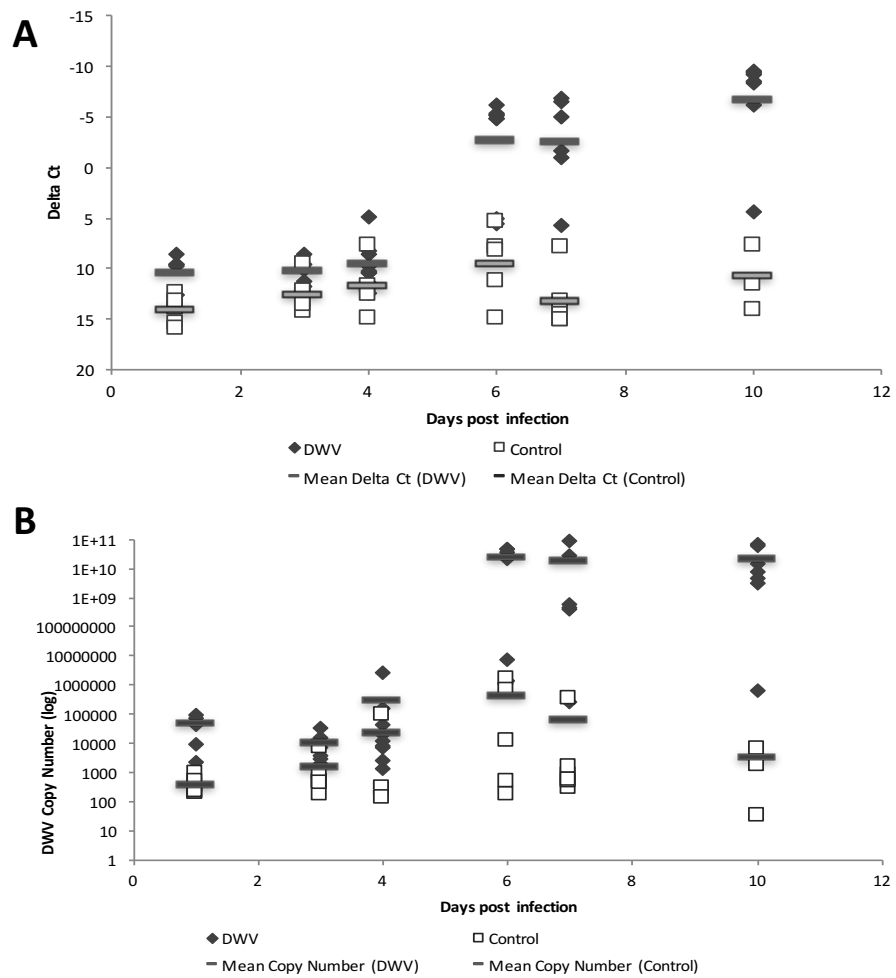
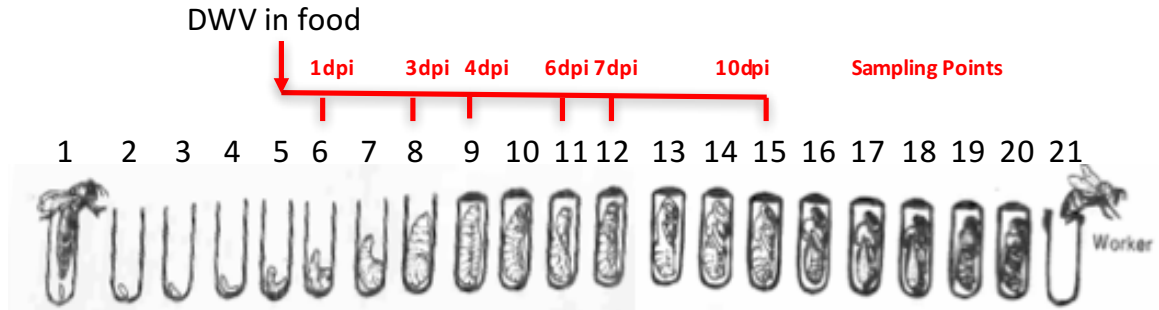


Figure 3.5. Time to Response - Quantification of DWV in honey bee pupae following inoculation via larval food. Single dose of 5.56×10^{13} DWV copies/larvae, given on day one of assay. Sampling at set time points during larval and pupal development. A. Delta Ct for Universal DWV primers, normalised to Actin. B. DWV copy number.

3.3.3. Quantification of DWV in honey bee pupae following inoculation via injection

3.3.3.1 Dose Response - Quantification of DWV in honey bee pupae following inoculation via injection

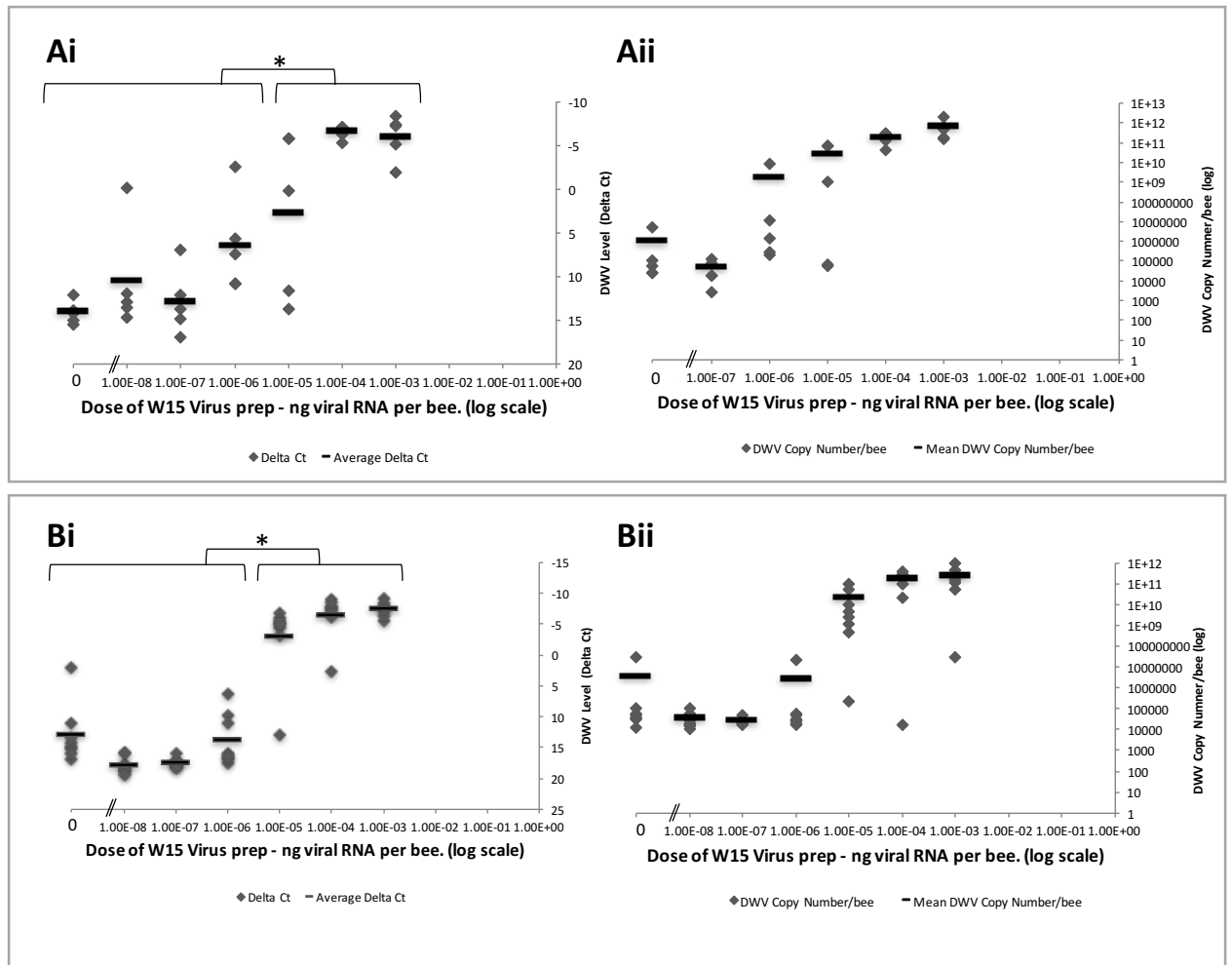


Figure 3.6 White eye pupae, day 12 to 13 from laying (4 days post capping) were injected with a single dose of DWV VP, doses ranged from 1.85×10^2 viral copies per individual, to 1.85×10^8 viral copies per individual. Sampling took place 4 days after inoculation. Results of two replicates shown. Results of Rep 1 are shown in Ai (delta Ct) and Aii (calculated DWV Copy Number per bee). Results of Rep2 are shown in Bi (delta Ct) and Bii (calculated DWV Copy Number per bee). Brackets show treatments found to give significantly different viral outcomes to the control injected pupae.

Batches of white eye pupae, day 12 to 13 from laying (4 days post capping) were injected with a range of doses of DWV VP, doses ranged from 1.85×10^2 viral copies

per individual, to 1.85×10^8 viral copies per individual. All individuals were sampled 4 days post injection, at the purple eye stage of development. Results of two replicate experiments are shown in figure 3.6. Injection of a dose of 1.85×10^7 viral copies was sufficient to produce a pronounced DWV infection in the majority of individuals (92% of $n=12$). However, the viral outcomes were more variable for doses between 1.85×10^6 viral copies and 1.85×10^7 viral copies per pupae. When a dose of 1.85×10^6 viral copies was given, 9/13 individuals (69%) were infected, and a dose of 1.85×10^5 viral copies resulted in an infection rate of 1/13 (8%). This result suggests that, for our VP, a dose of 1.85×10^7 viral copies per individual was sufficient to produce a symptomatic infection, but that there was some variation in susceptibility between individuals from the same colony. There was a significant effect of DWV dose on the viral load of injected individuals in both replicates, as determined by one way ANOVA, for rep 1 ($F(6,28)=14.28$, $p<0.05$) and rep 2 ($F(6,49)=73.95$, $p<0.05$). Post hoc analysis (Tukey HSD) revealed that, for both replicates, there were two distinct categories for viral outcome, with the doses of 1.85×10^7 viral copies per individual and greater having significantly higher viral loads upon sampling than the control and lower dose groups.

3.3.3.2. Establishing that ‘high DWV’ individuals go on to become symptomatic adults

In all of the above dose response experiments, the dose of DWV administered was not high enough to cause increased mortality in any of the treatment groups. The destructive nature of the sampling meant that, although we can state that DWV levels are in line with either asymptomatic or symptomatic individuals sampled from colonies, we cannot be sure that the ‘infected’ individual samples at ‘purple eye’ stage would have gone on to become symptomatic adults. To address this a repeat of the pupal injection experiment was conducted, with individuals given either a control injection or a dose of 1.85×10^6 viral copies per individual. This dose gave an infection rate, determined by qPCR at purple eye stage, of 69%. The injected pupae were left to

develop to adulthood in the incubator and then scored as having either normal or deformed wings, and normal or stunted abdomen. The adult bees in the DWV treatment group (n=50) were assessed as having deformed wings in 86% of individuals, and stunted abdomen in 72% of individuals. In contrast, the control injected group (n=25) exhibited wing deformities in 25% of cases and had no individuals (0%) with stunted abdomens. Independent t-tests show that the treatment and control groups differed significantly in terms of rate of deformity for both wings (t-value is -13.15435. The p-value is < 0.0005) and abdomen (t-value is -7.91021. The p-value is < 0.0005).

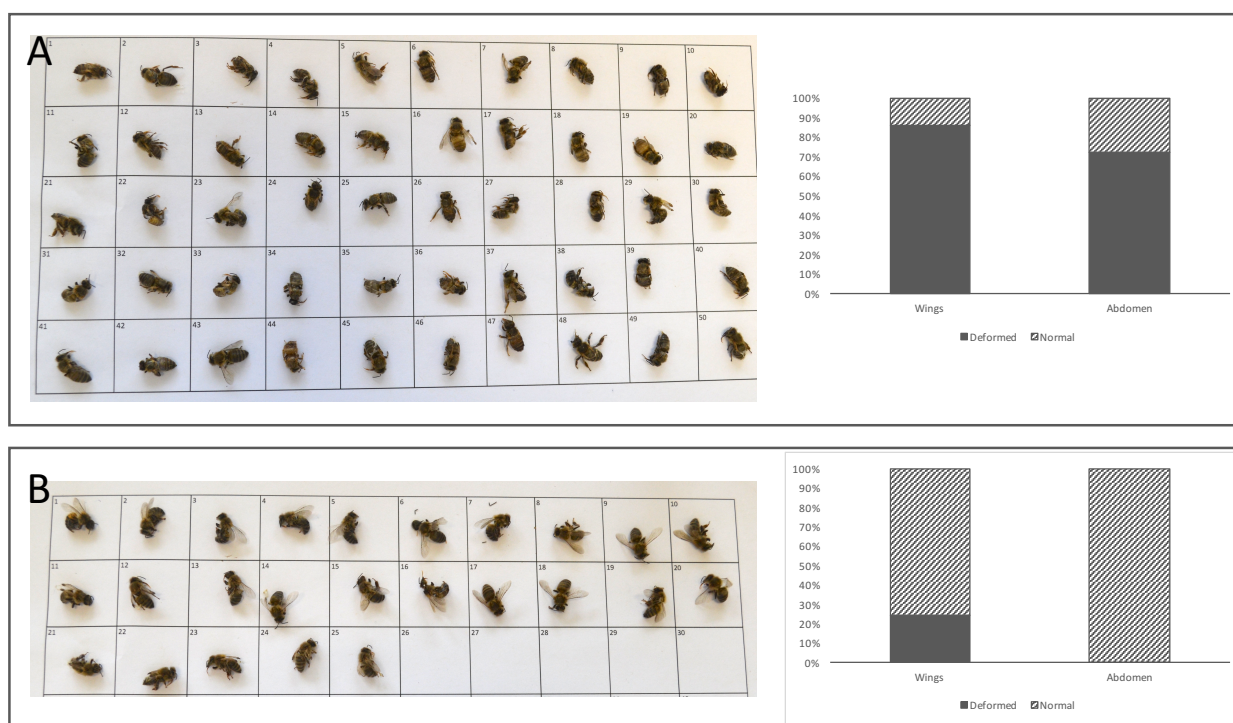


Figure 3.7. Honey bees injected with an infectious dose of DWV (1.85×10^6 viral copies) at white eye pupal stage and then left to develop in a humidity controlled incubator (33C, 90% humidity) to adulthood. Scored for wing deformity and stunted abdomen to allow assessment of symptom outcomes for DWV injected bees.

3.3.3.3. Time to Response - Quantification of DWV in honey bee pupae following inoculation via injection

Injection of DWV VP into ‘white eye’ pupae can be used to simulate DWV exposure through *Varroa* feeding, and the dose of DWV required to produce a symptomatic

infection is far lower when administered by direct injection than when administered orally. An experiment was done in which individual ‘white eye’ pupae from a single colony were given a single infectious dose of DWV (1.85×10^6 viral copies per individual) by injection, and then samples taken at regular intervals to reveal more about how DWV infection progresses in developing pupae.

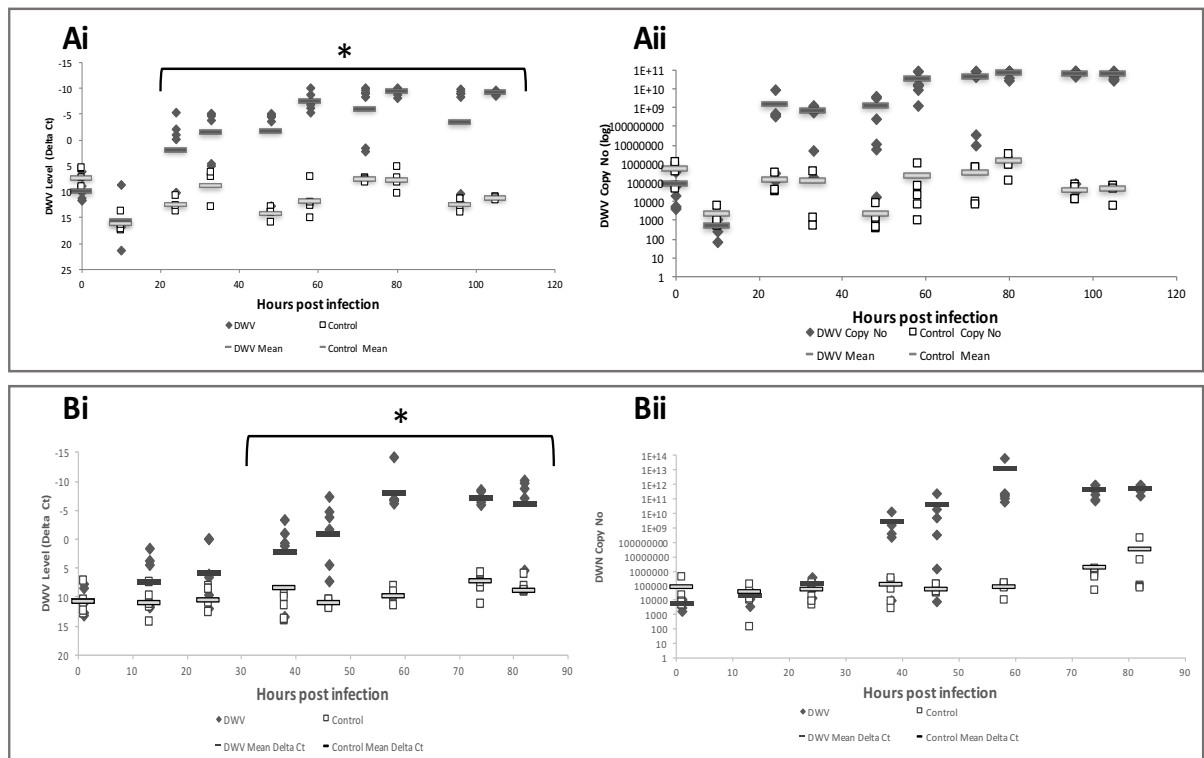


Figure 3.8. DWV injection into ‘white eye’ pupae. Dose of 1.85×10^6 viral copies per individual followed by sequential sampling up to purple eye stage. Results of two replicates shown. Results of Rep 1 are shown in Ai (delta Ct) and Aii (calculated DWV Copy Number per bee). Results of Rep2 are shown in Bi (delta Ct) and Bii (calculated DWV Copy Number per bee). Brackets show treatments found to give significantly different viral outcomes to the control injected pupae.

A two-way ANOVA was conducted that examined the effect of treatment (DWV or Control) and time on viral outcome. There was a statistically significant interaction between the effects treatment and sampling time on the level of DWV at sampling – Replicate 1, $F(9, 84) = 7.875, p < 0.005$. Replicate 2, $F(7, 72) = 11.985, p < 0.005$. In replicate 1, the DWV levels in both treatment groups were similar until the 24 h

sampling point, where the means for the DWV and Control differed significantly ($t(6)=-3.72$, $p < 0.05$). A similar pattern was observed in replicate 2, but here the means of the treatment groups did not differ significantly until the 38 h sampling point ($t(5)=3.08$, $p < 0.05$). This time period (24 to 38 hrs) was noticeably shorter than the time taken for DWV infections to become established in orally exposed bees (6 days/144 hours).

3.3.5 Localisation of DWV in honey bees using Stellaris probes (RNA-FISH) and confocal microscopy

Stellaris FISH (fluorescence *in situ* hybridization) is a RNA visualisation technique that permits detection, localisation, and quantification of individual RNA molecules within fixed, sectioned tissue using fluorescent microscopy. Stellaris FISH Probes are designed and produced as a set of multiple oligonucleotides with different sequences each with a fluorescent label that collectively bind along the same target transcript to produce a punctate signal.

The Stellaris probes cannot distinguish between RNA with high sequence homology, as discussed in chapter 2, so the probe set used was designed to detect both VDV-like and DWV-like capsid sequences.

This technique was used to reveal more about the time course of DWV infection discussed earlier in this chapter. A comparison of the localisation of the virus following experimental transmission, either via injection to mimic *Varroa* feeding or oral exposure to mimic horizontal transmission within the colony in the absence of *Varroa* was carried out.

3.3.3.1 Oral route of DWV exposure

RNA-FISH was done for samples taken from experiment 3.2.2.2 (time course assay)

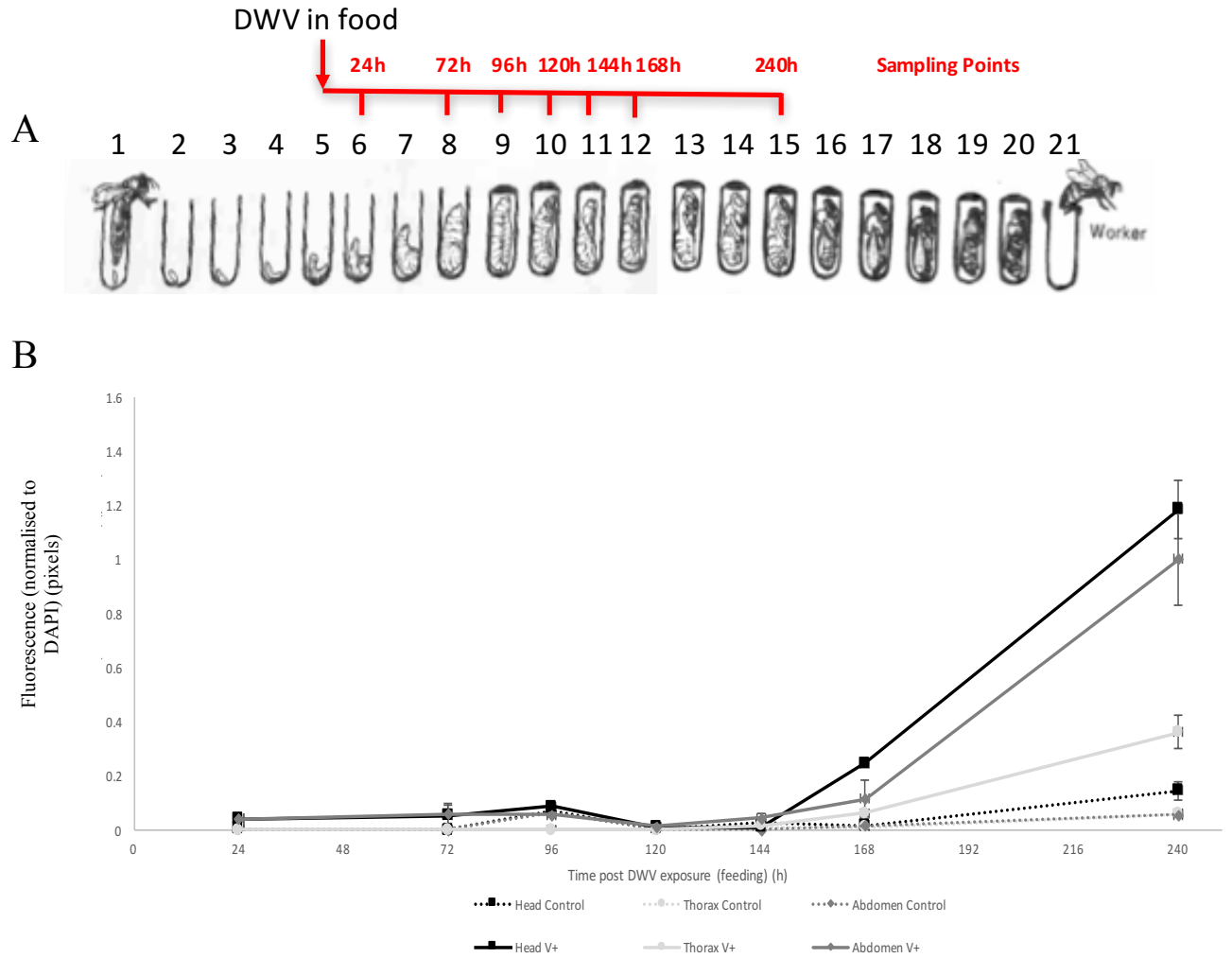
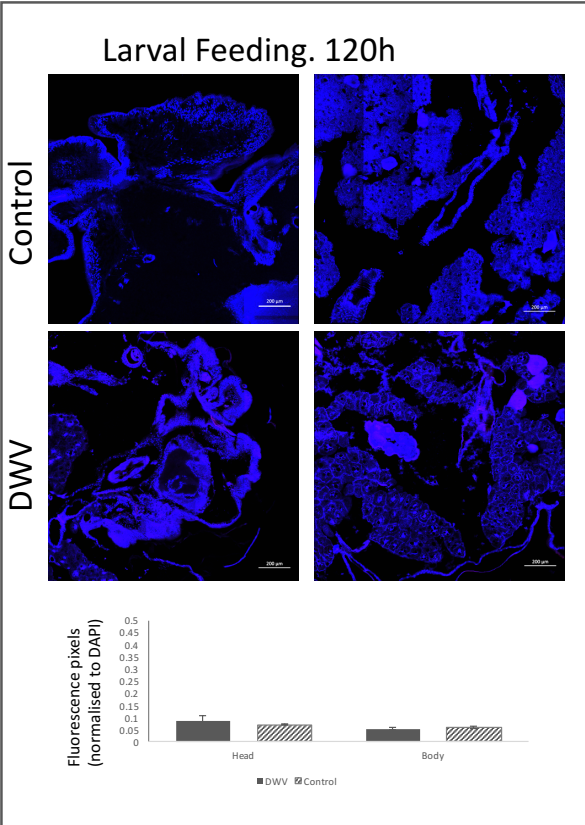
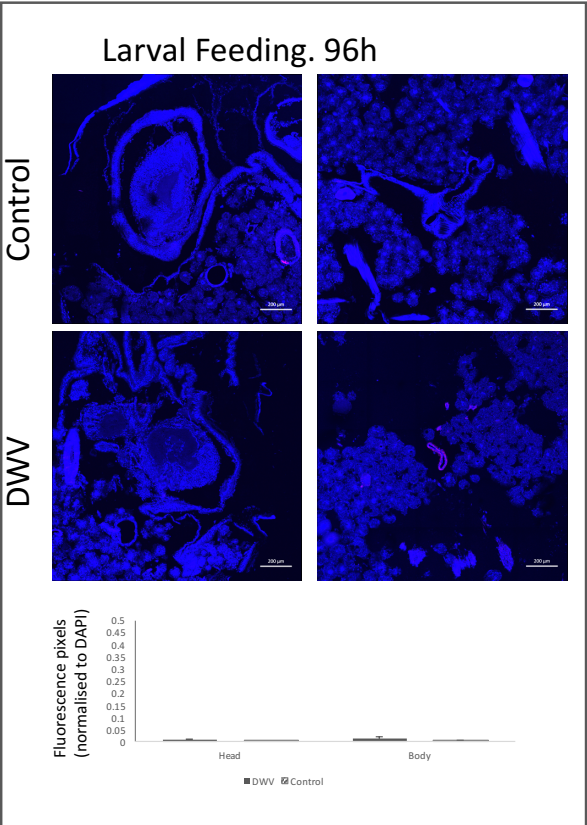
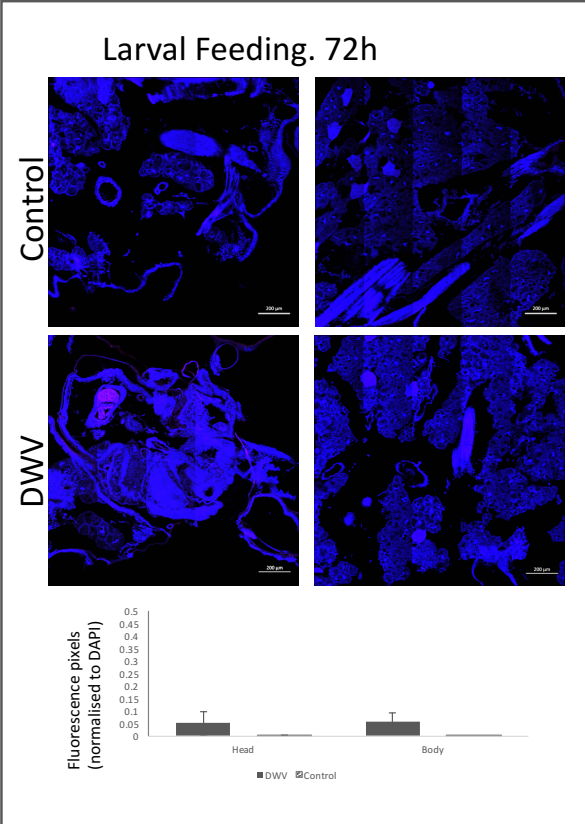
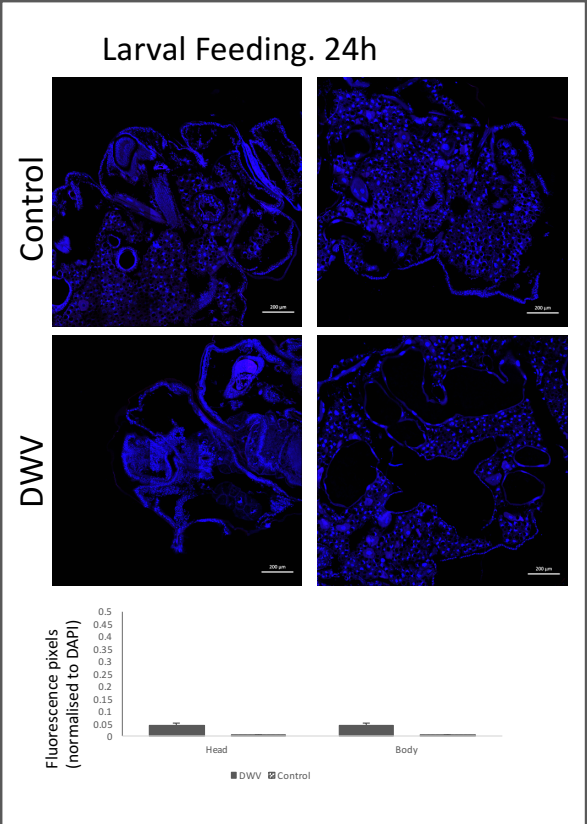


Figure 3.9 A. Sampling schedule for experiment. B. Fluorescence levels (normalised to DAPI levels to control for tissue density) for larvae fed a single dose of DWV (5.56×10^{13} viral copies per pupae, d5 post laying) and larvae fed on a virus free diet. Fluorescence measurements taken for different body segments - head, thorax and abdomen – from three individual insects per time point, per treatment group.

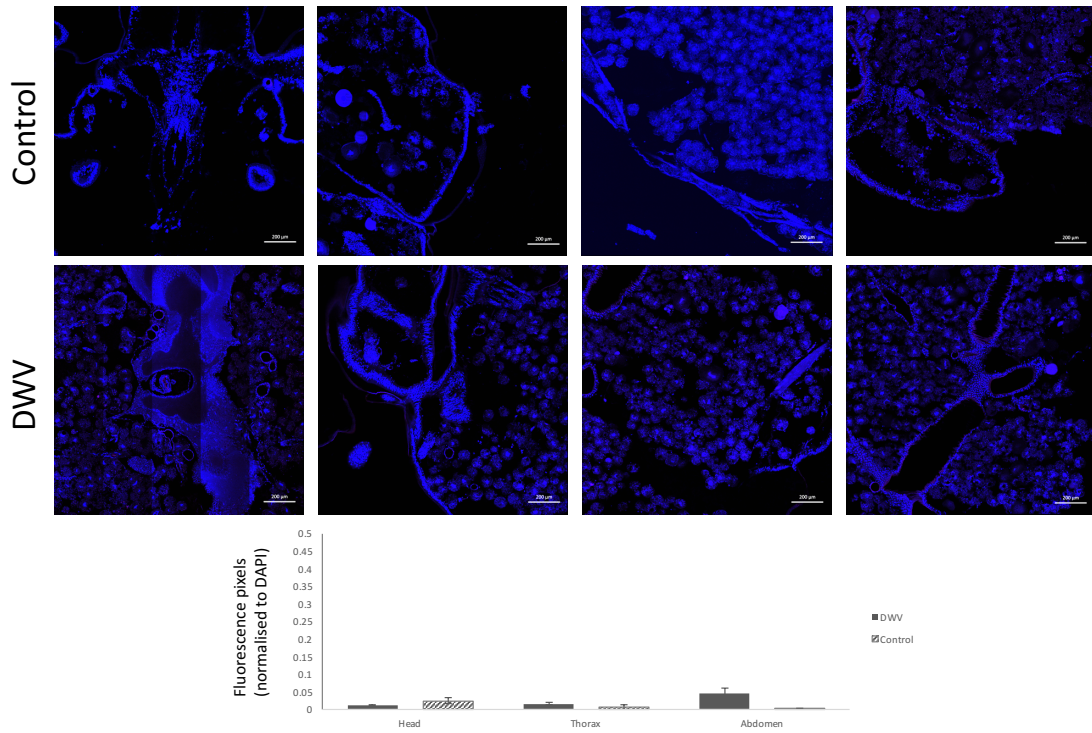
A two-way ANOVA was conducted that examined the effect of treatment (DWV feeding at 5 days post laying) and time from feeding on normalised fluorescence levels. There was a statistically significant interaction between the effects of treatment and sampling time on normalised fluorescence levels in the confocal images, $F(6, 166) =$

20.147, $p < 0.0005$. Post hoc testing (Tukey HSD) reveals that the treatment groups were not significantly different at the 24 h to 168 h sampling points, by the end of the experiment at 240 h the virus positive treatment group differed significantly from the control for all three body segments ($p < 0.0005$).

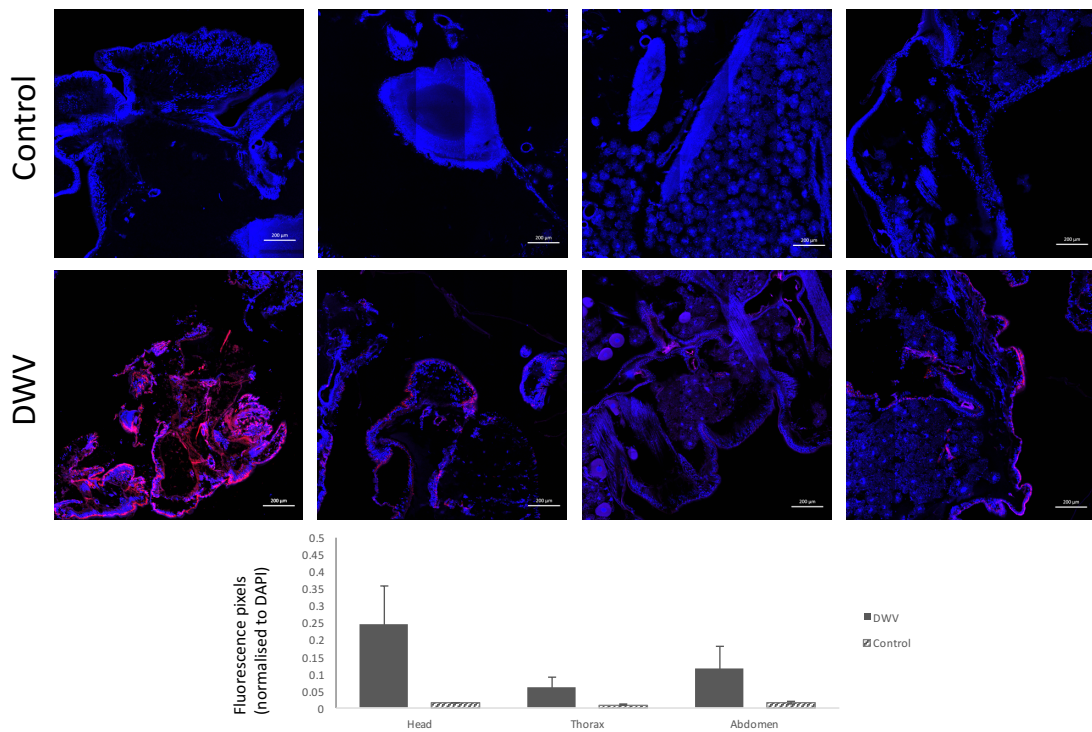
A closer examination of the confocal images revealed more about the progression of DWV infection in orally exposed honey bee larvae. Both the confocal images (fig 3.10) and the quantification of fluorescence (fig 3.9) show that levels of DWV in all body parts remain low for both treatment groups until after 168 h post DWV inoculation. At this point the larvae that were fed a high dose of DWV begin to show elevated levels of fluorescence in all body parts, indicating that DWV infection is progressing throughout the body. This time point corresponds with the transition from larvae to pupae. During this time period (0 to 3 days post capping) the honey bees are undergoing extensive metamorphosis, known as the propupal stage with the pupae developing within the larval skin. At 4 days post capping the bees undergo the fifth moult, when the external features of the adult honey bee become visible, although the majority of the internal organs are yet to form (Stell, 2012).



Larval Feeding. 144h



Larval Feeding. 168h



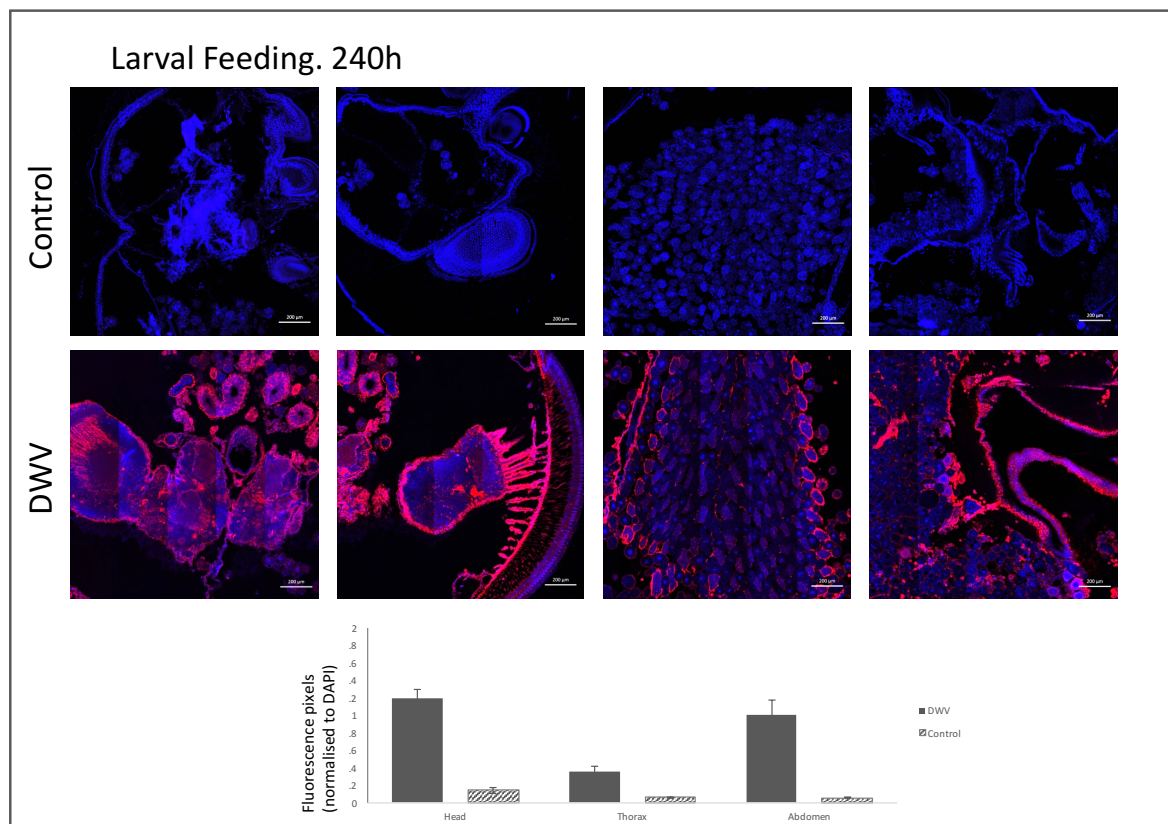


Figure 3.10 Confocal images of samples from larval feeding experiments, larvae fed a single dose of DWV (5.56×10^{13} viral copies per pupae, d5 post laying) and larvae fed on a virus free diet (Control). All slides hybridised with Stellaris RNA FISH probes designed to bind to DWV/VDV capsid sequences, set labelled with CAL Fluor® Red 590, and imaged at fixed laser settings on a Zeis 880 confocal microscope, 25x objective, 5x5 tile scan. Quantification of fluorescence was carried out using the Colour Pixel Counter plugin in Fiji (Schindelin, et al., 2012), with red fluorescence (DWV) being normalised to blue (DAPI) to control for tissue density.

3.3.3.2 Injection route of DWV exposure

Time course of infection experiments were also conducted for the direct injection route of DWV exposure, with these samples also being used for confocal imaging with Stellaris RNA FISH showing DWV localisation in developing pupal tissue.

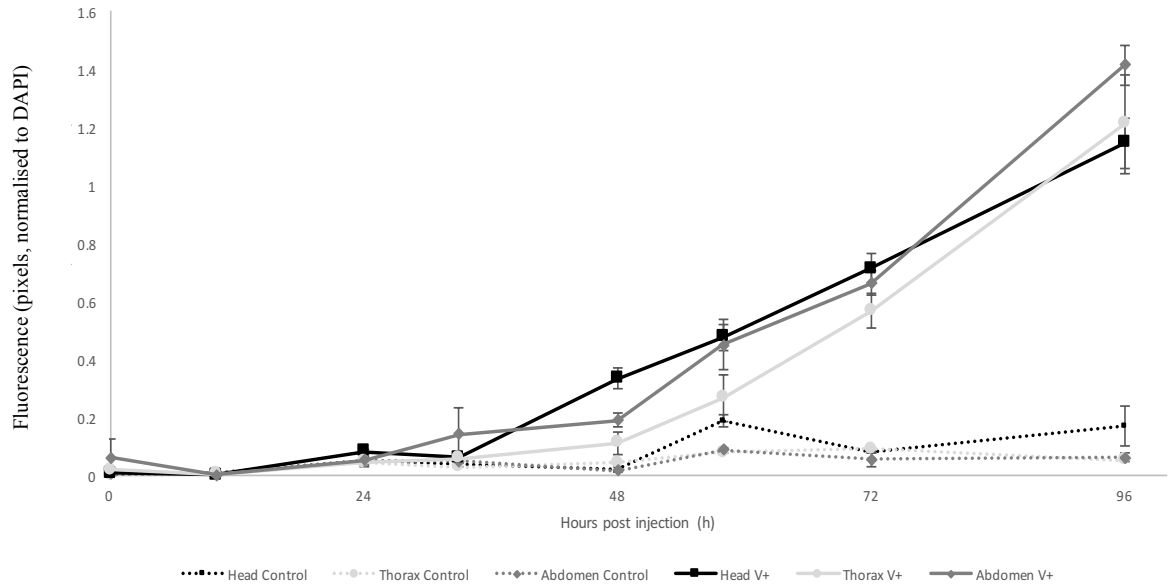
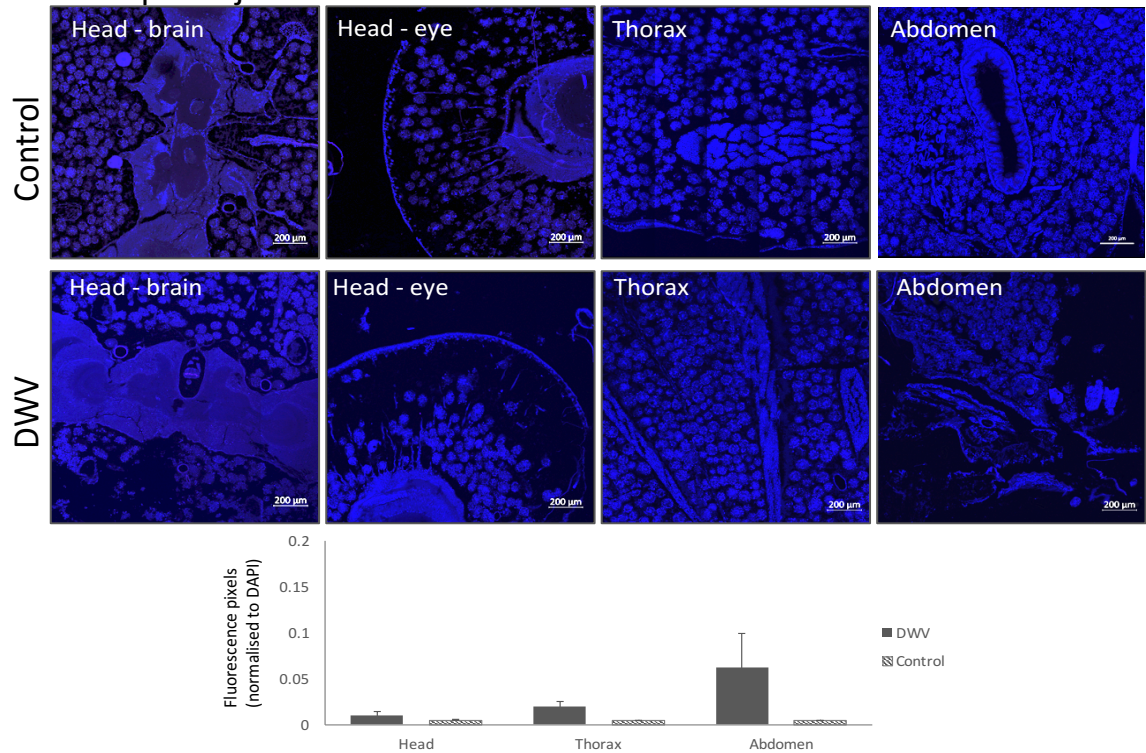


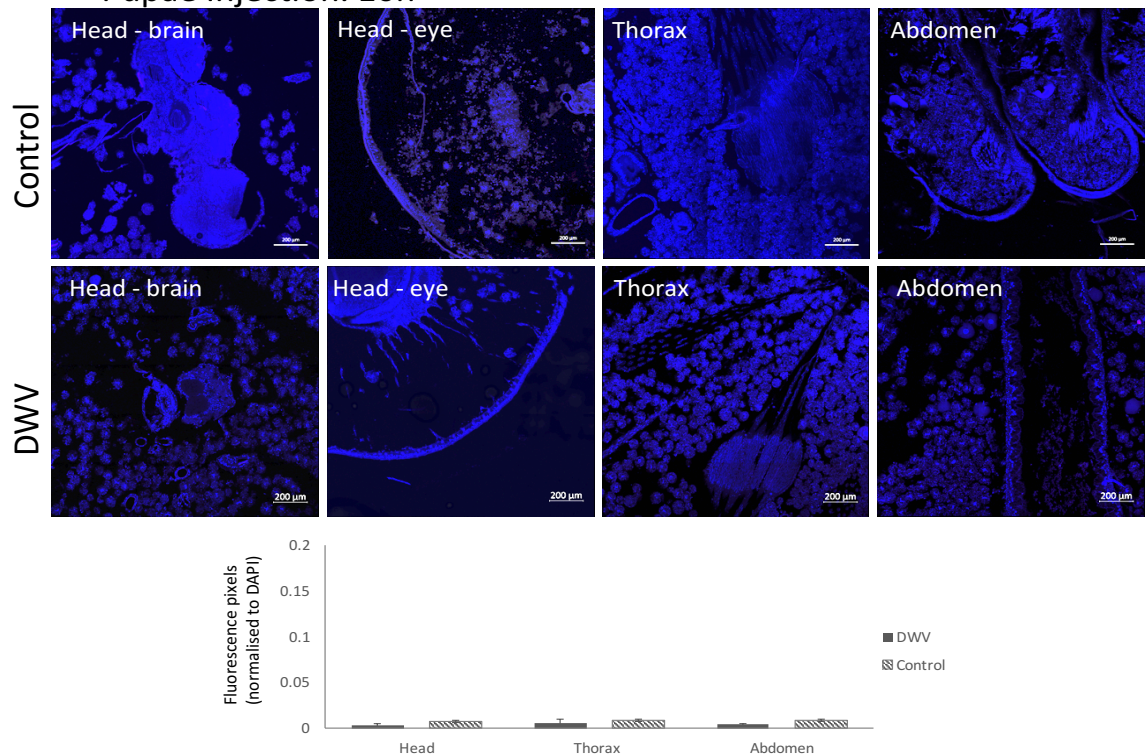
Figure 3.11 Fluorescence levels (normalised to DAPI levels to control for tissue density) for pupae injected with a single dose of DWV (1.85×10^6 viral copies per individual, white eye stage) and control pupae injected with PBS. Fluorescence measurements taken for different body segments - head, thorax and abdomen – from three individual insects per time point, per treatment group.

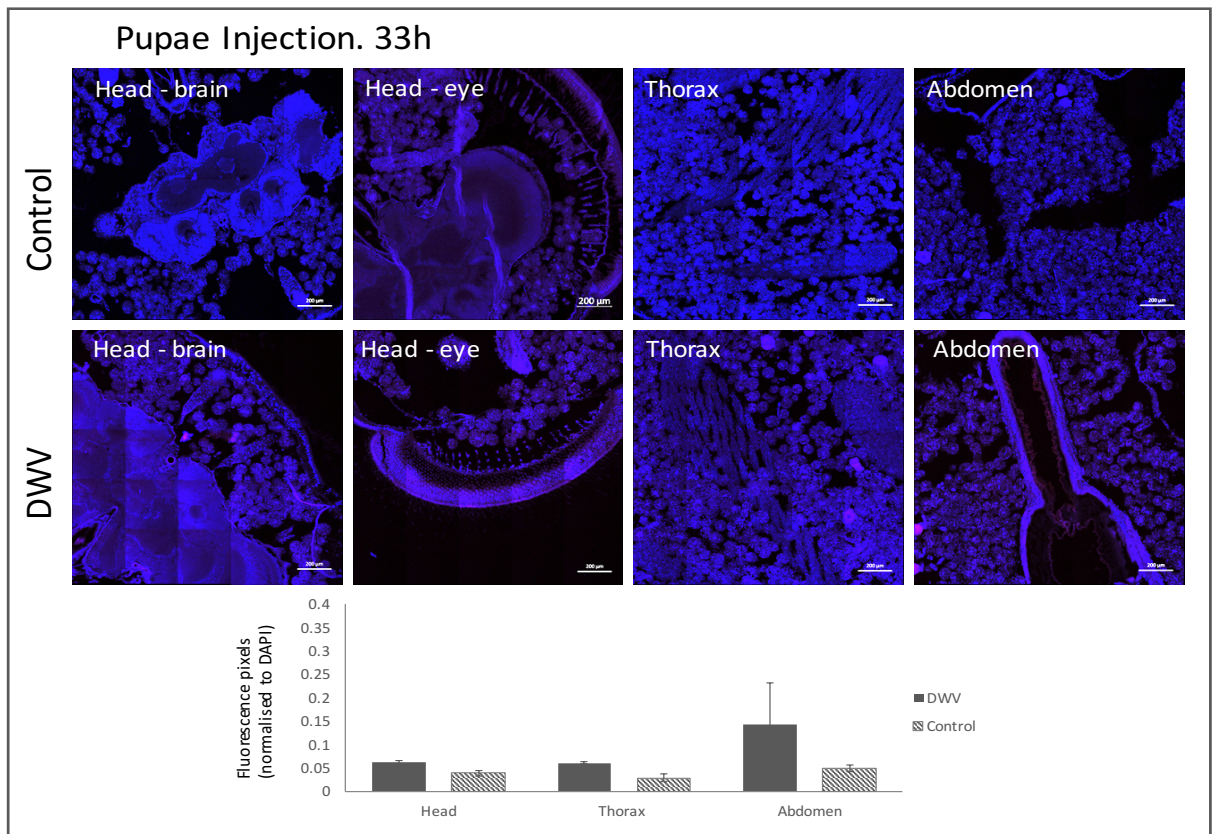
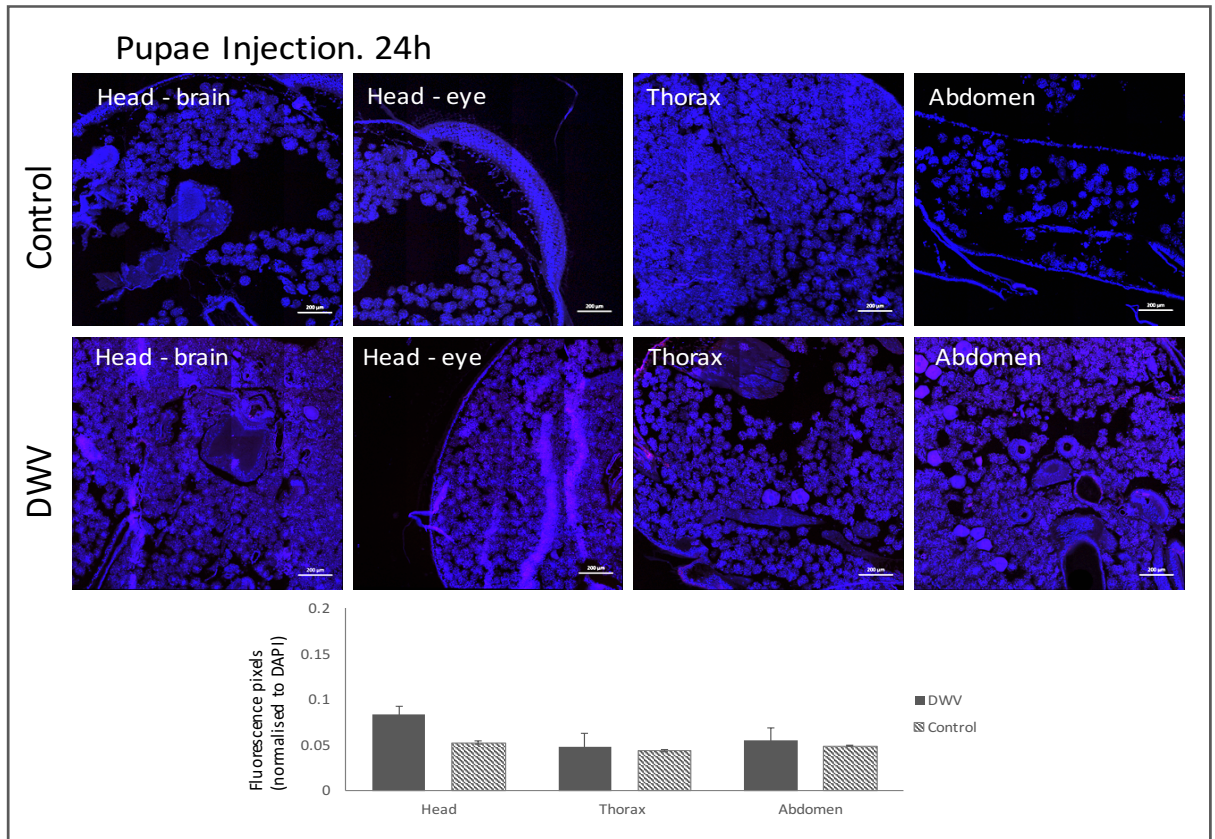
A two-way ANOVA was conducted that examined the effect of treatment (DWV injection at 3 days post capping) and time from injection on normalised fluorescence levels. There was a statistically significant interaction between the effects of treatment and sampling time on normalised fluorescence levels in the confocal images, $F(7, 254) = 84.898$, $p < 0.0005$. Post hoc testing (Tukey HSD) reveals that the treatment groups were not significantly different at the 0h to 33h sampling points, but from 48h after injection to the end of the sampling period, the virus positive treatment group differed significantly from the control ($p < 0.0005$).

Pupae Injection. 0h

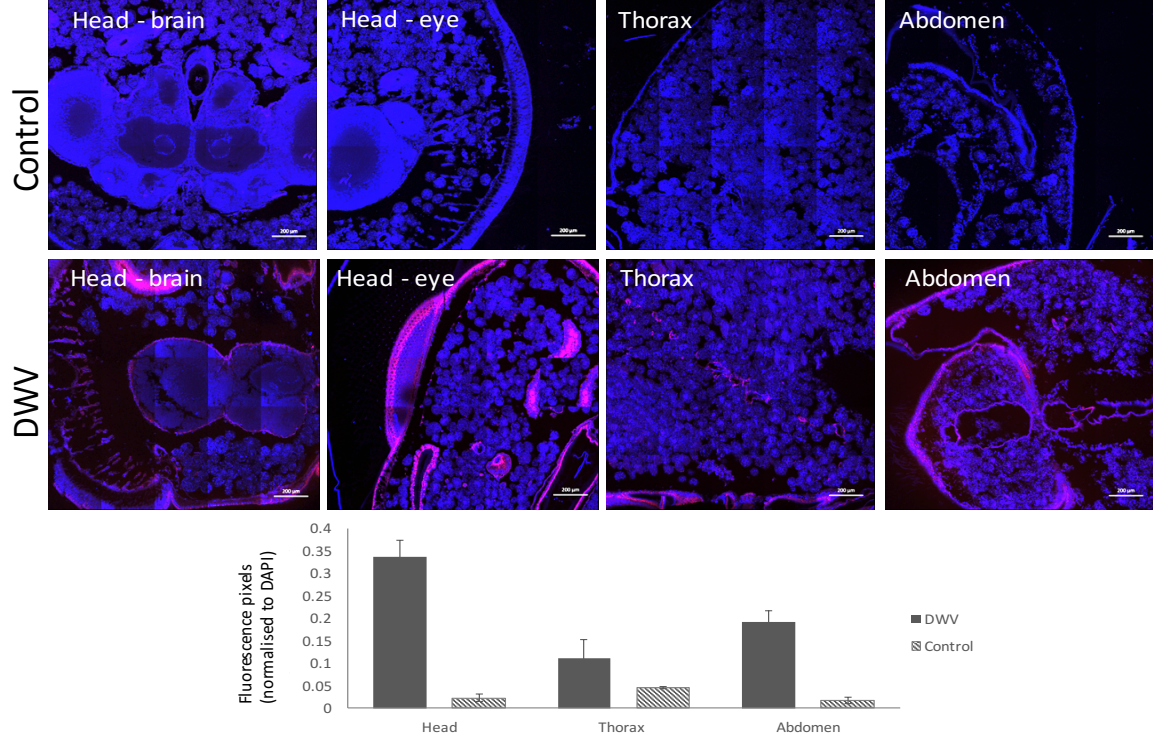


Pupae Injection. 10h

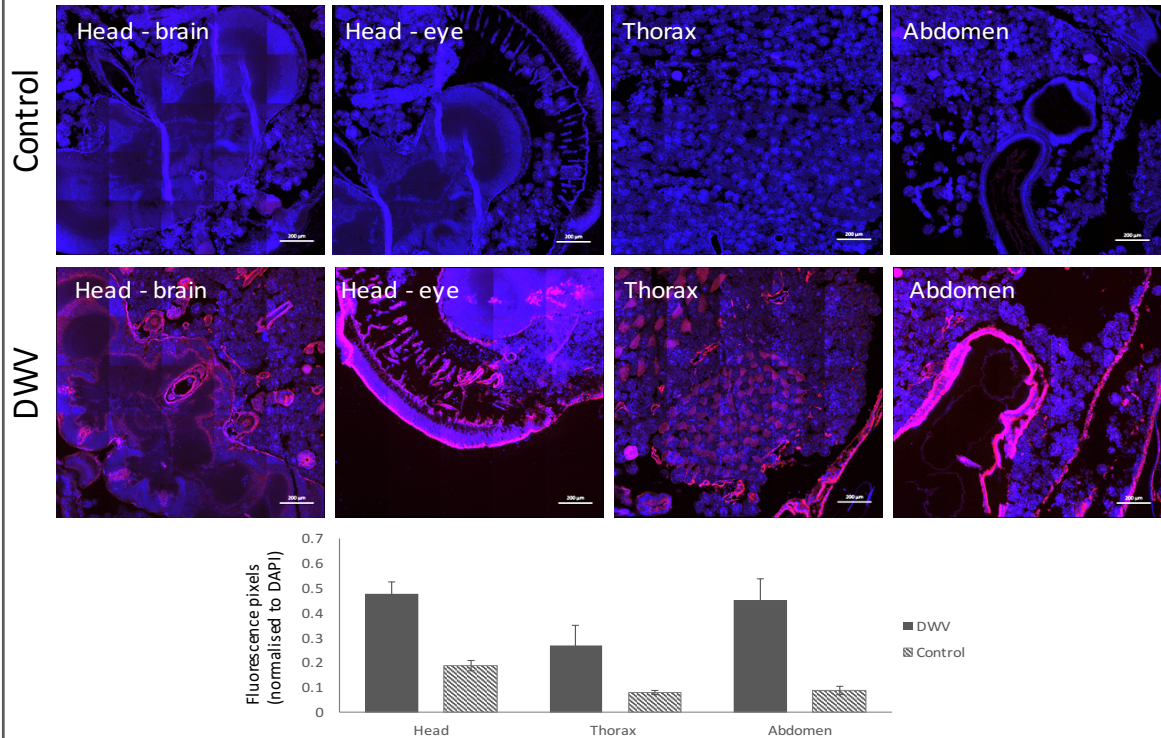




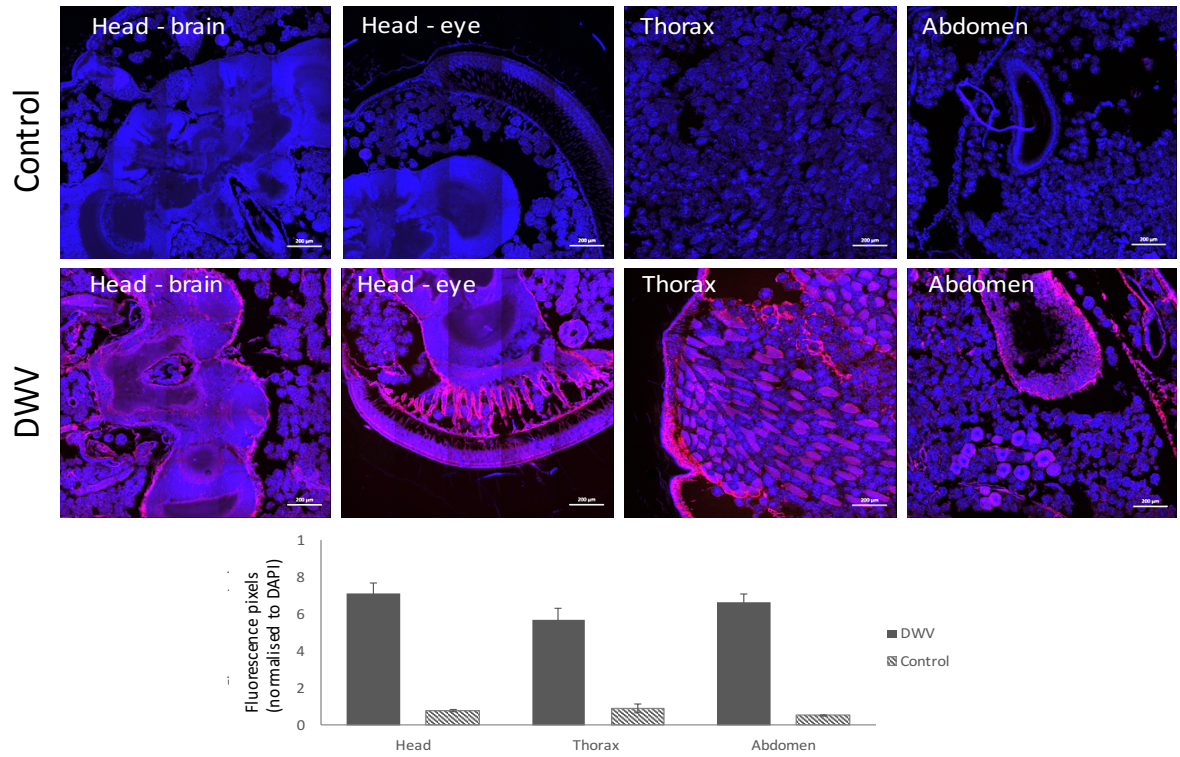
Pupae Injection. 48h



Pupae Injection. 58h



Pupae Injection. 72h



Pupae Injection. 96h

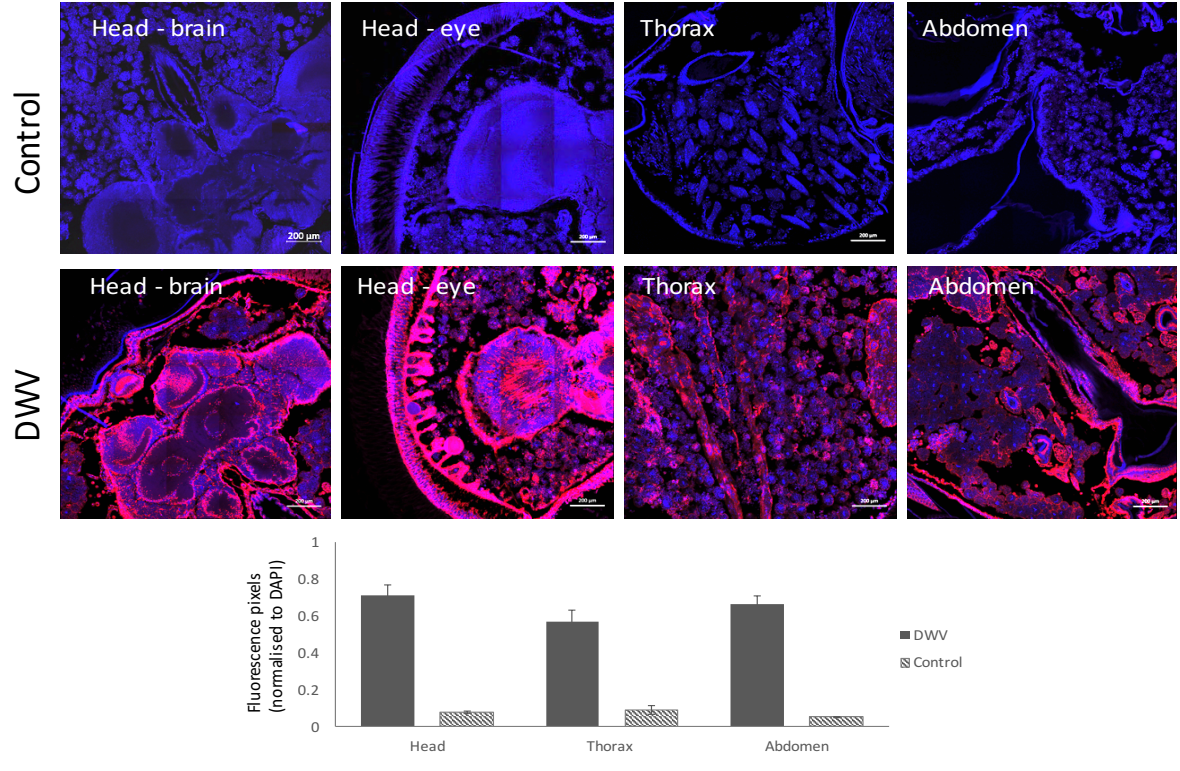


Figure 3.12 Confocal images of samples pupal injection experiments. Pupae injected with a single dose of DWV (1.85×10^6 viral copies per individual, d5 post laying) and larvae fed on a virus free diet (Control). All slides hybridised with Stellaris RNA FISH probes designed to bind to DWV/VDV capsid sequences, set labelled with CAL Fluor® Red 590, and imaged at fixed laser settings on a Zeis 880 confocal microscope, 25x objective, 5x5 tile scan. Quantification of fluorescence was carried out using the Colour Pixel Counter plugin in Fiji (Schindelin, et al., 2012), with red fluorescence (DWV) being normalised to blue (DAPI) to control for tissue density

A closer examination of the confocal images reveals more about the progression of DWV infection in injected honey bee pupae. The early sampling points (0h to 33h) show very low levels of fluorescence in both the control and DWV injected pupae. From 48h post injection onwards the levels of fluorescence are significantly greater in the DWV injected pupae than in those from the control group. A One-way ANOVA reveals that, at 48h post injection, there was a significant effect of body segment upon level of fluorescence in DWV injected pupae ($F(2, 22)=9.576$, $p=0.001$). Post hoc analysis reveals that fluorescence in the head (including the eye) differs from that in the thorax and abdomen. It appears that in injected pupae, DWV infection progresses fastest in the brain and eye tissue. However, by the next sampling point (58h) the infection appears to have progressed to a similar level in all of the body segments, with no difference in the measured fluorescence in the head, thorax and abdomen ($F(2,16)=2.527$, $p=0.107$).

3.4 Discussion

In honey bees unexposed to *Varroa* parasitism, DWV forms a low level, asymptomatic infection and is transmitted vertically from drones and queens, and orally between workers (Yue, *et al.*, 2006; de Miranda and Fries, 2008; Moeckel, *et al.*, 2011). DWV has been shown to reach higher levels and cause physical deformities and cognitive difficulties in honey bees when it is administered directly to the hemolymph of developing pupae, either via *Varroa* feeding (Gisder, *et al.*, 2009) or via experimental injection (Moeckel, *et al.*, 2011).

Until now, no work had been conducted that directly compared the dose of DWV required to cause a symptomatic or ‘high virus outcome’ infection in honey bees when administered either orally or via injection at the crucial larval and pupal stages of development. Here we have tested the hypothesis that DWV is more likely to cause a symptomatic infection when injected directly into the pupal hemolymph (mimicking *Varroa* feeding) than when it is dispensed orally to the developing larvae (mimicking oral transmission in brood food within the colony), as it gains access to a wider range of tissues in which to replicate and consequently is infectious at a far lower dose.

As described above, DWV infection can be seen as bimodal, with honey bees either exhibiting a low level asymptomatic infection or a high level symptomatic infection, with a clear distinction between ‘infected’ individuals with viral loads of around 10^9 or above, and asymptomatic individuals with significantly lower viral loads (Zioni, *et al.*, 2011; Ryabov, *et al.*, 2014a). Due to the destructive nature of the sampling required for this work we were unable to establish whether the individuals in these experiments would have gone on to exhibit DWV symptoms. However, I believe that there is enough evidence, both in the literature (Moeckel, *et al.*, 2011; Zioni, *et al.*, 2011; Ryabov, *et al.*, 2014a) and in section 3.3.3.2. of this chapter that we can confidently state that pupae with a viral load of greater than 10^8 copies of DWV are likely to go on to be symptomatic adult honey bees.

As expected, the oral dose of DWV required for a symptomatic infection when administered to *in vitro* reared honey bee larvae is dramatically higher than that required when the same DWV virus prep is inoculated directly into the pupal hemolymph. Injection of a dose of 1.85×10^7 viral copies was sufficient to produce a pronounced DWV infection in the majority of individuals, whereas 5.56×10^{13} viral copies/larvae was required to be mixed with larval food before any high viral load individuals were found. This dose of DWV is greater than the number of DWV genome equivalents that would be found within an entire symptomatic DWV infected honey bee. Whilst DWV has been detected in the brood food that workers feed to the developing larvae using RT-PCR (Yue and Genersch, 2005) the level of virus present has not yet been quantified. Still, it seems fair to assume that the quantity of DWV particles in this brood food would be significantly lower than the number present in an entire honey bee. This finding explains why, in *Varroa* naïve colonies, symptomatic DWV infected honey bees are rarely (if ever) present. However, brood food passed from nurse bees to larvae is not the only route of oral DWV transmission within a colony, workers exhibiting hygienic behaviour remove and cannibalize dead or diseased brood (Evans and Spivak, 2010; Moeckel, *et al.*, 2011) and any worker bee consuming a DWV infected pupae would receive a large dose of DWV, potentially as high as the 5.56×10^{13} viral copy threshold required for symptomatic infections in larvae. This infection route becomes more likely as the *Varroa* numbers in a colony increase, with any worker bees that avoided *Varroa* parasitism during their development (and were therefore unlikely to have a symptomatic DWV infection upon emergence) becoming infected through their hygienic duties as nurse bees. Up to 100% of sampled workers in a collapsing, late season colony can have high level DWV infections (see Chapter 4, figure 4.8) in spite of not having visible DWV associated deformities. This consumption of heavily DWV infected brood could shorten the lifespan and reduce the foraging ability of these late season bees (Dainat, *et al.*, 2012a; Benaets, *et al.*, 2017).

The experiments described above also reveal more about the time course of DWV infection in honey bees exposed to infectious doses of DWV, either orally as larvae or

via injection as pupae. This time period required for bees given an infectious dose of DWV via injection to have a DWV infection detectable for qPCR (24 to 38 hrs) was noticeably shorter than the time taken for DWV infections to become established in orally exposed bees (6 days/144 hours). With both modes of transmission, the transition from low to high DWV load comes at the same time as the virus becomes detectable in the brain and developing compound eye, plus the muscle tissue in the developing thorax and the epithelial tissues of the mid and hind gut (Stell, 2012). In injected pupae, there is no physical barrier between the virus and the vulnerable tissues, and infection progresses quickly, detectable by qPCR 24 to 38 h after inoculation. However, in orally exposed individuals, the gut may form an effective barrier to infection (DeGrandi-Hoffman and Chen, 2015), and in the feeding experiments in this chapter DWV infection was not able to progress, even with a very high oral dose of DWV, until the larval gut tissue was broken down following pupation (Stell, 2012).

It has been shown by other research groups that a symptomatic DWV infection can be characterized by the presence of DWV in the head tissue of the adult bee (Moeckel, *et al.*, 2011) but the RNA-FISH imaging experiment described in this chapter reveals more about where DWV localises in a honey bee pupae during the early stages of an infection. 48 h after injection with an infectious dose of DWV the virus particles are seen to be accumulating in the developing compound eye, the layer of cells surrounding the brain tissue, the salivary glands and mouth parts and in the epithelial cells of the mid and hind gut. It appears that these tissues are most vulnerable to DWV infection once the virus has been injected or escaped from the digestive tract. By the 58 h post injection sampling point, the virus particles are also shown to be present within the brain tissue and in the muscle tissue of the thorax. As the infection progresses through the 72 h and 96 h sampling points the levels of fluorescence throughout the honey bee tissue increase dramatically, until there appears to be little tissue selectivity for virus replication, with all body parts becoming heavily infected. As described above, the orally administered virus takes longer to establish an infection, but once the breakthrough has occurred the progression of infection and tissues affected appear to be the same as in injected individuals.

A pupal injection experiment was carried out to check that individuals given the dose of DWV virus prep that caused high viral load (as shown by qPCR) actually went on to develop the characteristic DWV symptoms of deformed wings and stunted abdomen (Results section, 3.3.3.2). The proportion of DWV injected bees recorded as having a stunted abdomen was very close to the one predicted by the earlier qPCR results (72% observed, 69% predicted), with no control injected bees exhibiting a stunted abdomen, allowing us to be confident that the sampled individuals with high viral loads would have gone on to be symptomatic adults. However, the rate of deformed wings was higher than expected, (86% observed, 69% predicted) and 24% of the control group also showed some wing deformities. It has been shown that chilling of brood can lead to the emergence of individuals with wing deformities in both honey bees (Wang, et al., 2016) and in *Apis cerana cerana* (Zhou, et al., 2011). It is possible, that despite efforts to keep pupae warm during collection and injections, that the slight cooling that occurs whilst handling the white eye pupae could lead to wing deformities. It is therefore necessary to look for both deformed wings and a stunted abdomen when categorising an individual as having symptomatic DWV.

Chapter 4. How does the spread of the *Varroa* mite alter the prevalence and population diversity of DWV-like viruses?

4.1 Introduction

RNA viruses characteristically exhibit high levels of genetic variation, a trait that is thought to enable them to respond to the changes in host immune defences as part of the host-pathogen coevolutionary ‘arms race’ (Obbard and Dudas, 2014). The high mutation rates, rearrangement by genetic recombination, short replication times and high replication yields exhibited by RNA viruses result in them existing as dynamic mutant ‘swarms’, known as viral quasispecies (Domingo and Holland, 1997). This genetic variation is created and maintained within infected cells (Del Portillo, *et al.*, 2011; Leitch and McLauchlan, 2013) and organisms during viral infections and outbreaks (Domingo, *et al.*, 2012; Gire, *et al.*, 2014). It has been proposed that existing as diverse quasispecies allows these pathogens to quickly overcome the challenges of a new environmental niche, such as the colonisation of a new host species, through the selection of the most fit distributions for the new environment (Domingo, *et al.*, 2012). RNA viral populations have also been shown to persist as a number of related master variants, each with a ‘swarm’ of randomly generated mutants and it is this model that has been applied to explain the diversity seen in honey bee viruses such as DWV and IAPV (Palacios, *et al.*, 2008; Mordecai, *et al.*, 2016b).

As described in Chapter 1 of this thesis, DWV is a single-stranded, positive-sense, picorna like virus which is very prevalent in honey bee colonies globally (Lanzi, *et al.*, 2006). DWV is closely related to VDV-1 and KV (Fujiyuki, *et al.*, 2004; Ongus, *et al.*, 2004). The strains have been shown to recombine (Moore, *et al.*, 2011), and are considered to form a viral quasispecies, or variants of the same species complex. It has been suggested that it may be this genetic diversity which has allowed DWV-like viruses to capitalise on the global spread of the *Varroa* mite, transitioning from a comparatively benign, generally asymptomatic infection to a major honey bee pathogen. This theory has been supported by recent research which shows a shift in the predominant strain of the virus when bees

have been exposed to *Varroa* parasitism, both on a broad landscape level upon the introduction of *Varroa* to Hawaii (Martin, *et al.*, 2012) and at the level of individual honey bees (Ryabov, *et al.*, 2014).

Work published by our research group indicates that a virulent recombinant form of DWV is able to replicate to high levels when it is injected directly into honey bee haemolymph, either by *Varroa* feeding or by experimental injection. It is this recombinant strain of the disease (with VDV-like capsid sequences and DWV-like non-structural regions) which was found to be present as a near clonal population in bees with high virus levels, both *Varroa* exposed pupae and symptomatic adults. In contrast, *Varroa* naïve, low virus individuals were found to have a very diverse population of DWV-like viruses (Ryabov, *et al.*, 2014).

This narrowing in viral diversity in bees parasitized by *Varroa* mites was effectively shown on a larger scale by Martin *et al.* (2012) in their work with Hawaiian honey bees. The introduction of the mite to the islands significantly increased the prevalence of DWV, and this increase in viral prevalence was accompanied by a severe reduction in the diversity of DWV-like viruses, leading to a single predominant viral strain. Although the techniques used in the Hawaii study did not allow the identification of the strain that was able to capitalise on the introduction of *Varroa*, this work along with the study conducted on our Warwickshire bees (Ryabov, *et al.*, 2014) has triggered a lot of interest in the honey bee research community in how the spread of the *Varroa* mite alters the prevalence and population diversity of DWV-like viruses (McMahon, *et al.*, 2016; Mordecai, *et al.*, 2016a; Mordecai, *et al.*, 2016b; Brettell, *et al.*, 2017).

Subsequent published work has established that *Varroa* mites do have an impact on the strains and diversity of DWV-like viruses within a honey bee colony, but there is not yet an agreement on the identity of a single ‘virulent strain’ or ‘master variant’ of DWV (Table 4.1). This suggests that the picture is more complex, and a number of hypotheses have been proposed to explain the results found by different research groups.

In their work looking at the DWV population in an apiary of isolated honey bees near Swindon, Wiltshire UK that appear to be able to tolerate high *Varroa* loads without colony losses (Mordecai, *et al.*, 2016a) found that DWV-like viruses were present at high levels but did not appear to cause any of the ill effects usually associated with *Varroa* transmitted DWV. They reported that this particular apiary was dominated by VDV-like viruses (termed DWV type B by the paper) and that DWV-like or recombinant strains were absent. It was suggested that a phenomenon called superinfection-exclusion, in which a pre-existing viral infection is able to prevent a closely related virus forming a secondary infection (Folimonova, 2012), may explain how bees in this apiary are able to survive. This would mean that VDV (or DWV type B) is an avirulent strain of the virus and that bees infected with this strain are protected from the more harmful of virulent forms of the virus (DWV-like strains/DWV type A). Mordecai, *et al.*, (2016b) have also identified another ‘master variant’ of DWV, through Illumina sequencing and phylogenetic analysis of honey bee samples from an apiary in Devon, UK, and have named this variant DWV type C. The two colonies sampled for this paper contained a mixture of DWV type A (DWV-like) and type C, with very low levels of DWV type B (or VDV-1), while one of the colonies – which subsequently died out over winter – contained a higher proportion of DWV type C. Mordecai *et al.* (2016b) concluded that this could indicate that DWV type C is a virulent variant, and that the low levels of VDV-1 in the colonies mean that they are not benefiting from the protection of superinfection exclusion observed in other bees from the same study. The authors do allow that direct manipulation experiments, such as those conducted by (Ryabov, *et al.*, 2014) would be needed to determine whether the DWV type C strain is particularly virulent.

A follow up study to the Martin *et al.* (2012) study in Hawaii described above (Brettell, *et al.*, 2017) used next generation sequencing to compare the DWV genomes in six Hawaiian samples (three colonies, each containing a single deformed bee and 30 asymptomatic bees) in an attempt to determine which variant is responsible for wing deformities in infected bees. DWV-like (type A) viruses dominated in all samples, with one exception – a mixed virus population found in asymptomatic bees from one colony with a slight leaning towards VDV-like viruses (DWV type B). DWV-type C was only

found in one asymptomatic sample, alongside DWV type A. The paper concludes that is no single DWV strain associated with wing deformity; but that many variants have the potential to cause symptoms in honey bees.

The results reported in another recent UK study (McMahon, *et al.*, 2016) appear to contradict the conclusions drawn by Mordecai *et al.* (2016 b). This work involved the experimental injection of DWV-like virus extracts into adult honey bees and to observe the impact on mortality. The field-derived virus strains used for inoculation were categorised as either DWV-like (Type A) or VDV-like (Type B) or an experimentally created mixture of the two (A and B). Illumina sequencing was used to ascertain the predominant viral sequence in the inocula. Injection with all strains of the virus significantly reduced honey bee lifespan in comparison with the control group, and bees exposed to VDV-like viruses (type B) or a mixed injection had their median survival rates reduced still further than those injected with DWV (type A). Analysis of the sampled bees also found extensive evidence for recombination between DWV and VDV-1 (types A and B), but no evidence of DWV type C (the strain that was suggested to be virulent by Mordecai *et al.* (2016 b)). McMahon *et al.* (2016) went on to analyse samples from a field survey of honey bee foragers at 25 sites across the UK. They found no significant difference between the prevalence of DWV and VDV-1, but some differences in the spatial distribution of the two, with DWV being more uniformly spread across the UK and VDV more commonly found in samples from the South East of the country. It was also found that the percentage of sampled bees infected with both DWV and VDV-1 was greater than would be predicted by chance.

The fact that these studies have reached different conclusions is an indication that we don't yet fully understand how and why the introduction of *Varroa* mites has transformed the population of DWV-like viruses within honey bee colonies. Some of the inconsistencies may be explained by differences in methodology – experimental manipulation vs environmental sampling, pooled samples vs individual honey bees, sampling at different life stages (pupae vs newly emerged adults vs foragers) or geographic location see table 4.1. It is also difficult to draw firm conclusions from small

sample sizes and single apiary experiments, but the current cost of NGS is prohibitive so larger scale studies are not presently feasible.

Table 4.1 Summary of current research into DWV/VDV-1 virulent strains.

Publication(s)	Methodology/ sampling	Life Stage	Strain identification	Virulent Strain?	Comments
<i>Ryabov et al. 2014</i>	Experimental manipulation and small scale environmental sampling. Individual samples	Pupae, Symptomatic and asymptomatic adults	Sanger sequencing of cloned PCR products Strain specific qPCR Illumina	DWV/VDV recombinant	
<i>Martin et al. 2012</i>	Large scale environmental sampling. Pooled samples	Asymptomatic adults	High Resolution Melt	DWV (determined by later sequencing (Brettell et al. 2017))	A change in viral diversity is shown, but no claim as to the dominant strain before or after <i>Varroa</i> infestation.
<i>Mordecai et al. (2016 a and b)</i>	Small scale environmental sampling. Pooled samples.	Asymptomatic adults	High Resolution Melt Illumina	DWV or DWV-type C	Suggest that VDV-1 is avirulent and forms a protective infection.
<i>McMahon et al. 2016</i>	Experimental manipulation and large scale environmental sampling. Pooled samples.	Adults (injected as pupae) Pooled foragers.	Sanger sequencing of cloned PCR products Strain specific qPCR Illumina	VDV-1 caused greater mortality in injected bees.	

The one firm conclusion that we can draw is that further research is required to reveal how the interaction between honey bees, *Varroa* and DWV is changing the viral landscape. It seems likely that there is a certain element of the viral genome that confers a selective advantage when the disease is transmitted by *Varroa*, as this would create a strong selection pressure leading to the large shifts in predominant viral strain that have repeatedly been observed upon the introduction of *Varroa* mites - either to a *Varroa* naïve

area or individual bee. However, as until very recently a reverse genetic system for DWV had proved elusive, researchers have been working with DWV preparations extracted from environmental samples (see table 4.1). These virus preps can be sequenced and categorized as DWV-like or VDV-like, but there has been no way to create a ‘pure’ preparation of a single strain, and no way to manipulate and combine viral coding regions to test their effect on the virulence of the virus. An infectious clone of DWV has just been published (Lamp, *et al.*, 2016) and it is hoped that this will allow accurate, repeatable manipulation of the virus, shedding light on any elements of the DWV genome that confer virulence to the virus when it is injected into the honey bee hemolymph.

However, there is clearly still much to be gained by informed collection and analysis of environmental honey bee samples. The inconsistencies in the currently published work are intriguing and, undoubtedly, further analysis of how the DWV-like virus population is influenced by *Varroa* transmission will help to clear up and explain these contradictions and inform future molecular investigation of the viral genome.

In this chapter I describe the impact of *Varroa* parasitisation on the population of DWV-like viruses, both in a single apiary in Warwickshire, and on a larger scale, in an area at the forefront of *Varroa* expansion in the UK. The aim of this work is to track any changes in the level of DWV, and in the most prevalent strain of the virus, as the *Varroa* mites become established in the area.

A collaboration with Science and Advice for Scottish Agriculture (SASA) and beekeepers from the Ardnamurchan Peninsula, an area at the active infestation expansion front for *Varroa* in the UK has allowed a longitudinal study of the DWV-like viruses present in apiaries in the area. Ardnamurchan is a 50-square-mile peninsula in the area of Lochaber, in the Western Highlands of Scotland. It is noted for its remoteness, with the only access to most of the peninsula being one single track road. There is no beekeeping on the majority of the peninsula but there are some apiaries closer to the hamlets of Acharacle and Strontian, on the eastern or mainland end of the headland. *Varroa* mites were first found in the UK on 4th April 1992 in Devon, and spread fairly quickly from the south

coast throughout England and Wales as the high density of apiaries made it very difficult to prevent phoretic mites being carried between colonies. *Varroa* mites were first reported in Southern Scotland in the mid 2000's (Grey, *et al.*, 2008) but the spread of the parasite throughout the remote and sparsely populated North and West of the country has been slow, as is shown by the map published by SASA in 2012 following a national survey (Figure 4.1). The first *Varroa* mites were reported in Ardnamurchan in 2013 (Fiona Hight (SASA) and Kate Atchley (local beekeeper), personal correspondence). The spread of the mites since that point has been very slow due to beekeepers treating intensively and selling or removing affected colonies, so there are still some apiaries in the area that appear to be *Varroa* free. For the research presented in this thesis, samples of honey bee brood collected from a number of apiaries in the area at the end of the 2014, 2015 and 2016 beekeeping seasons have been examined using strain specific qPCR quantification of the levels of DWV-like and VDV-like capsid and polymerase sequences in individual honey bee pupae. This allowed us to build up a picture of the levels of DWV and of any major shifts in the mixture of viral strains.

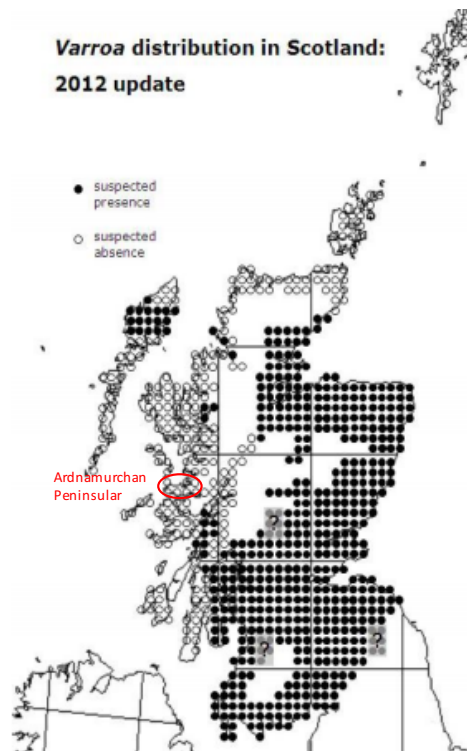


Figure 4.1 This map shows the areas of reported presence and assumed absence of *Varroa destructor* in Scottish honeybees in 2012. The map has been adapted from (Sunderland, et al., 2013). The Ardnamurchan Peninsula is circled in red, and had no reported incidence of *Varroa* mites in 2012.

I also conducted a year long study in a local apiary (maintained on the University of Warwick campus (Gibbet Hill), Coventry UK), sampling regularly in order to quantify whether the level and strain of DWV-like viruses changed within a colony if the *Varroa* mite population was allowed to grow unchecked. This is of interest because many beekeepers do not treat their colonies for *Varroa* until the end of the beekeeping season, when the *Varroa* levels are regularly very high, meaning that winter bees are often infected with high levels of DWV (Martin, 2001; Martin, *et al.*, 2010; Locke, *et al.*, 2017). If a virulent strain of DWV has accumulated within the colony during the season, late treatment for *Varroa* would mean that the overwintering bees would be infected with this more harmful strain, with the possible consequence of shortening their lifespans and therefore making overwinter colony loss more likely. If the colony is strong enough to make it through the winter in spite of this disease burden, then the strain of DWV carried

by these overwinter bees is likely to be passed to the new brood and therefore persist within the colony.

Throughout the 2015 season two adjacent colonies in the Gibbet Hill apiary were managed to give very different *Varroa* populations – with one (‘White’ Colony) being intensively treated with vaporised oxalic acid and thymol to keep mite numbers low, and the other (‘Blue’ colony) was untreated, allowing mites to accumulate to high levels. Samples of adult bees and brood were taken throughout the season and *Varroa* levels were closely monitored.

The aim of this chapter was to reveal more about how transmission of DWV by *Varroa* impacts the mixture of DWV-like strains present within honey bees, both in *Varroa* naïve bees and in a ‘normal’ UK colony.

4.2 Methods

4.2.1 Analysis of predominant strain of DWV in the Ardnamurchan Peninsula, an area at the forefront of *Varroa* expansion in the UK.

4.2.1.1 Ardnamurchan Peninsula Sampling

Brood samples were collected in mid-September by Fiona Highet (SASA) and the collaborating beekeepers. They were collected as excised sections of capped brood, flash frozen and then stored at -80°C until processing. Individual pupae were removed from the comb whilst still frozen on dry ice and each cell was thoroughly checked for *Varroa* mites. RNA extraction and clean up was carried out according to the methods in Chapter 2 of this thesis (Section 2.6).

4.2.1.2 Ardnamurchan Peninsula – Analysis of DWV levels and predominant strain.

Total RNA was analysed for 8 individual pupae, per colony, per year. Levels of DWV and predominant strains were identified using qPCR (as in Chapter 2, Section 2.6.2). Results were confirmed using nested PCR detection of DWV, VDV1 and their recombinants (as in Chapter 2, Section 2.6.1) and cloning and sequencing of the PCR products. Two colonies were selected for this confirmation cloning, Baker2-2015 (*Varroa* free) and SB2-2015 (High *Varroa*). 27 clones from each colony were sequenced (9 clones from each of three individual pupae), and the sequences were assessed as being either DWV-like, VDV-like or recombinants by BLAST search results and Clustal alignment (Altschul, *et al.*, 1990; Thompson, *et al.*, 1997; Goujon, *et al.*, 2010).

Samples were also analysed using a ‘High Resolution Melt’ (HRM) technique, as described in (Martin, *et al.*, 2012) and in (Mordecai, *et al.*, 2016a). High resolution melt analysis is a novel, post-PCR analysis technique, allowing the identification of variation in nucleic acid amplicons. It relies on the detection of small differences in PCR melting curves, generated by differences in the length, GC content and heterozygosity of the

amplicons (Cousins, *et al.*, 2012). Unfortunately, the HRM assay did not distinguish between the strains of virus present in the samples. This is regrettable, as HRM analysis would have provided an excellent visual representation and numerical quantification of the diversity of DWV-like viruses in the samples (as in Martin et al. (2012) supplementary information

<http://science.sciencemag.org/content/sci/suppl/2012/06/07/336.6086.1304.DC1/Martin.SM.pdf>). However, our qPCR assay has been shown to reliably identify the predominant viral strain in a sample, giving the same outcome as Illumina sequencing and Sanger sequencing of cloned amplicons in our work published in (Ryabov, *et al.*, 2014). Further analysis of the HRM assay and strain specific qPCR results is provided in the discussion section of this chapter (Section 4.3).

Name of Beekeeper (anonymised by JF)	Beebase ID	Sample ID	Aplary Co-ordinates	2014	2015	2016	Comments	Varroa status
KA	38126	M1	NM68346926	✓			sold 2015	Since 2012
		M2	NM68346926	✓			sold 2015	Since 2012
		B3	NM8115962423	✓	✓	✓		Since 2015
		B4	NM8115962423	✓	✓	✓		Since 2015
		K1	NM7169472460	✓			sold 2015	not yet found
		K2	NM7169472460	✓			sold 2015	not yet found
SB	53766	SB1	NM6779068885	✓	✓		Overwinter loss 2015/16	since 2012
		SB2	NM6779068885	✓			Overwinter loss 2014/15	since 2012
JPB	46564	Baker 2	NM8192763880	✓	✓	✓		Since 2016
JM		D1	see copy map		✓	✓		Since 2016
		D2			✓	✓		Since 2012
DM		K3	see copy map			✓		Varroa Free

Table 4.2 Details of samples collected from the Ardnamurchan Peninsula 2014 to 2016. Samples shaded in red were known to have Varroa mites in the colony at the time of sampling. Those in grey were thought to be Varroa free, having been thoroughly inspected for the presence of Varroa throughout each sampling season.

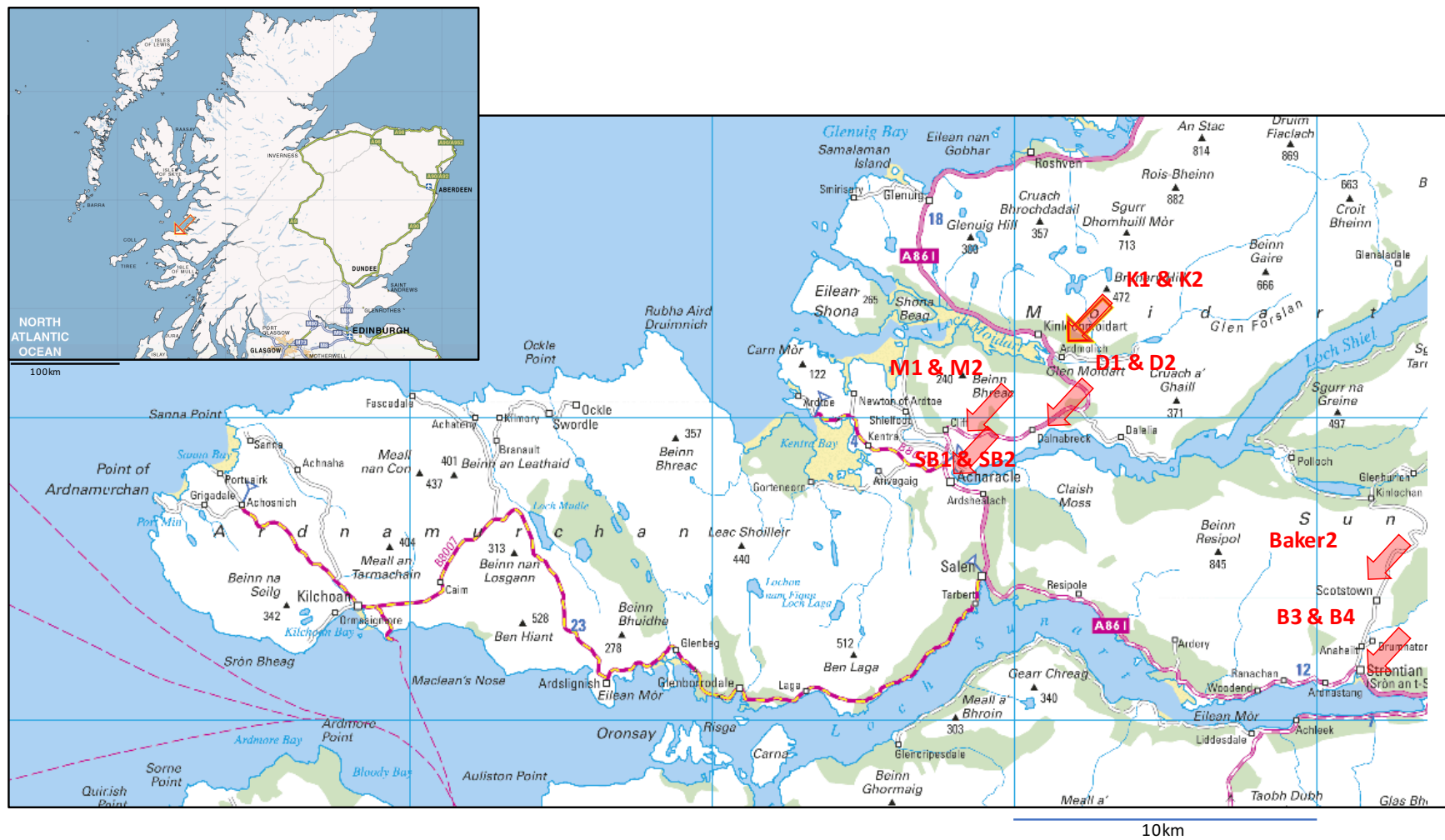


Figure 4.2. Map of the Ardnamurchan Peninsula showing locations of sampled apiaries. Map courtesy of Ordnance Survey, via streetmap.co.uk

4.2.2 Single Apiary Experiment, analysis of the change in predominant strain of DWV in a single colony as *Varroa* infestation takes hold

4.2.2.1 Single Apiary Experiment - Beekeeping and Sampling

Two colonies were selected from a single apiary - located on the University of Warwick campus – in the spring of 2015. Both colonies had been created from a split of a single, strong colony during the summer of 2014 and had been treated to reduce *Varroa* numbers using Apiguard (thymol, Vita Bee Health, UK) in September 2014, according to manufacturer's instructions.

Samples of adult bees and of capped pupae were taken at the start of the 2015 season (20/4/2015) and then one colony (hereafter named 'Treated' or T) was treated with vaporised oxalic acid (Api-Bioxal) (using a Sublimox vapouriser, Icko Apiculture, Italy. Details of treatment outlined here <https://theapiarist.org/sublimox/>) to remove phoretic *Varroa* mites (Maggi, *et al.*, 2017; Papezikova, *et al.*, 2017). Treatments took place on the 20th, 25th and 30th April 2015 and then again on 1st, 5th and 10th June. The oxalic acid does not remove any mites within capped cells so 3 repeat treatments were carried out each time to kill any recently emerged *Varroa*. At the end of the season Apiguard was applied to the T colony. The other colony (hereafter named 'Untreated' or U) did not receive any *Varroa* treatments in the 2015 season.

Samples of adult bees were taken monthly during the early summer (April, May, June) and then every two weeks throughout July to November as the *Varroa* numbers began to increase in colony U. At each sampling point, a three day *Varroa* drop was collected and counted, and this figure was used to calculate the *Varroa* load for each colony. *Varroa* load was calculated using the Natural Mite Mortality method outlined by The National Bee Unit, UK, as described in Methods Chapter 2.1.1) and checked using their online calculator (<http://www.nationalbeeunit.com/public/BeeDiseases/varroaCalculator.cfm?>).

Samples were stored at -80 °C until processing. RNA was extracted and cleaned up according to the methods in Chapter 2 (2.5) of this thesis.

4.2.2.2 Single Apiary Experiment - Analysis of DWV levels and predominant strain.

Total RNA was analysed for 10 adult bees per sampling point. Levels of DWV and predominant strains were identified using qPCR (as in Chapter 2, Section 2.7.2). Results were confirmed using nested PCR detection of DWV, VDV1 and their recombinants (as in Chapter 2, Section 2.6.1).

4.3 Results

4.3.1. Analysis of predominant strain of DWV in the Ardnamurchan Peninsula, an area at the forefront of *Varroa* expansion in the UK

4.3.1.1. DWV levels and strain in sampled bees

Samples were categorised according to a). year of sample collection (figure 4.3 A) or b). number of years of known *Varroa* infestation for the sampled apiary, with the year of collection being disregarded (figure 4.3 B). Total DWV copy number was determined for each sampled individual using qPCR. This allowed us to build an accurate picture of the levels of DWV-like virus' in honey bees in the region as *Varroa* became more established.

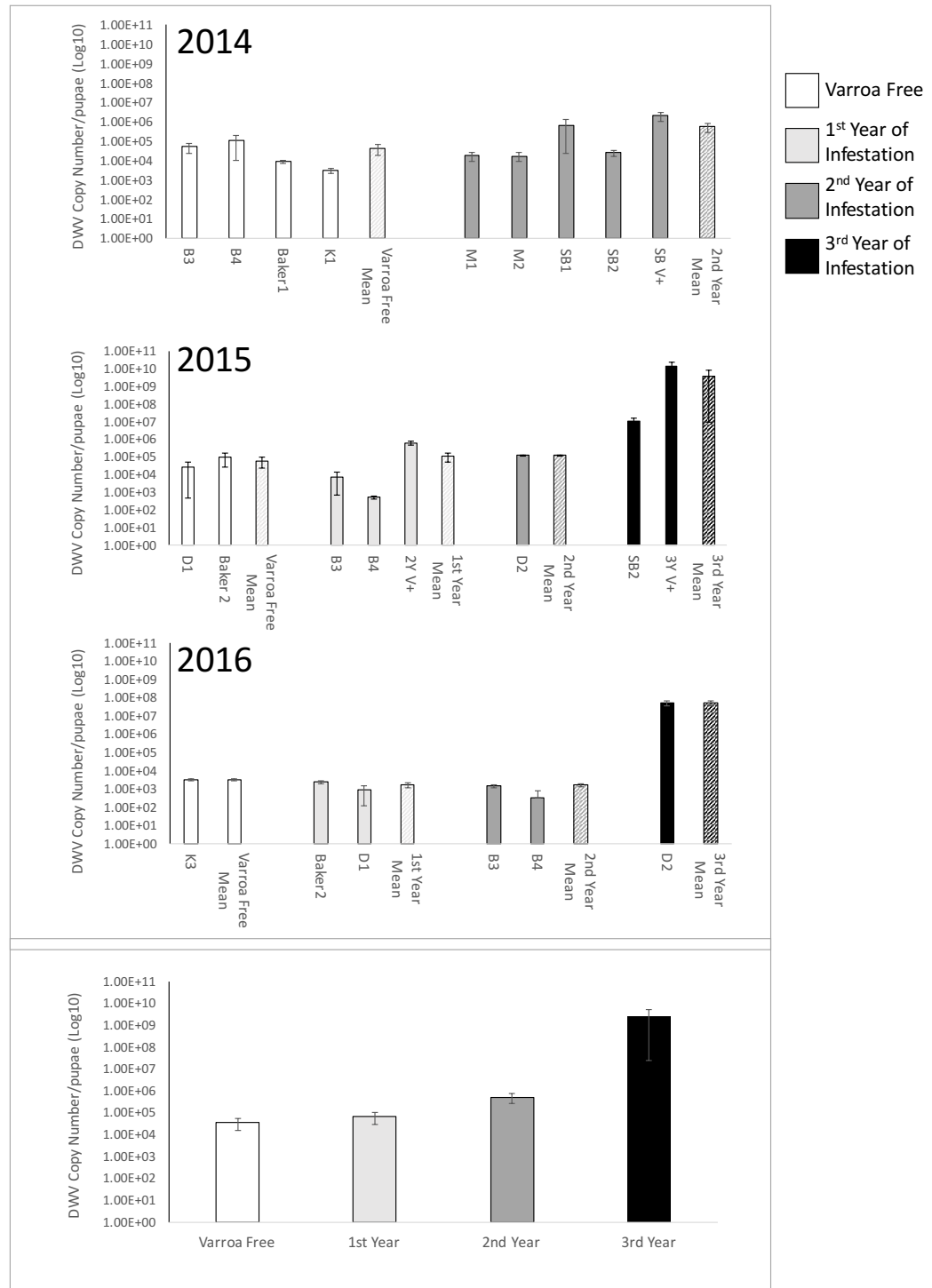
A

Figure 4.3. A DWV copy number per bee pupae, as measured by qPCR, from individual bees samples at different apiary sites in the Ardnamurchan Peninsula during the natural colonisation of Varroa mites along the Peninsula from 2014 to 2016. Samples were categorised according to whether they came from apiaries with or without Varroa. Apiaries with Varroa were further categorised according to the number of years since the infestation first occurred. Hashed bars represent category means. DWV copy number per bee shown on a Log10 scale. **B.** Combined means for the three sampling years.

There was no statistically significant difference in DWV copy number/pupae for samples collected in 2014, 2015 and 2016, as determined by one-way ANOVA ($F(2,161) = 0.855$, $p=0.427$). We had hypothesised that DWV levels would increase in the sampling period, as *Varroa* mites became more established in the Ardnamurchan Peninsula (as was observed in Hawaii after the introduction of *Varroa* (Martin, *et al.*, 2012)), however this was not the case.

The detailed information provided by the Ardnamurchan beekeepers on the presence or absence of *Varroa* in each sampled apiary allows us to also look at DWV levels as a product of the number of years for which *Varroa* have been present in the apiary, rather than solely separating samples by the year in which they were collected. Hence we can categorise samples as being ‘*Varroa* Free’, ‘1st Year of *Varroa* Infestation’, ‘2nd Year of *Varroa* Infestation’ or ‘3rd Year of *Varroa* Infestation’ (Figure 4.3, B). There was a statistically significant difference in DWV copy number/pupae for samples collected in the 1st, 2nd and 3rd year of *Varroa* infestation and samples from *Varroa* free apiaries, as determined by one-way ANOVA ($F(3,190) = 2.686$, $p=0.048$). Post hoc analysis (Tukey HSD) reveals that samples from year of *Varroa* infestation 3 have a significantly different load of DWV virus, when compared to the samples from *Varroa* naïve apiaries, or from apiaries where *Varroa* are less established.

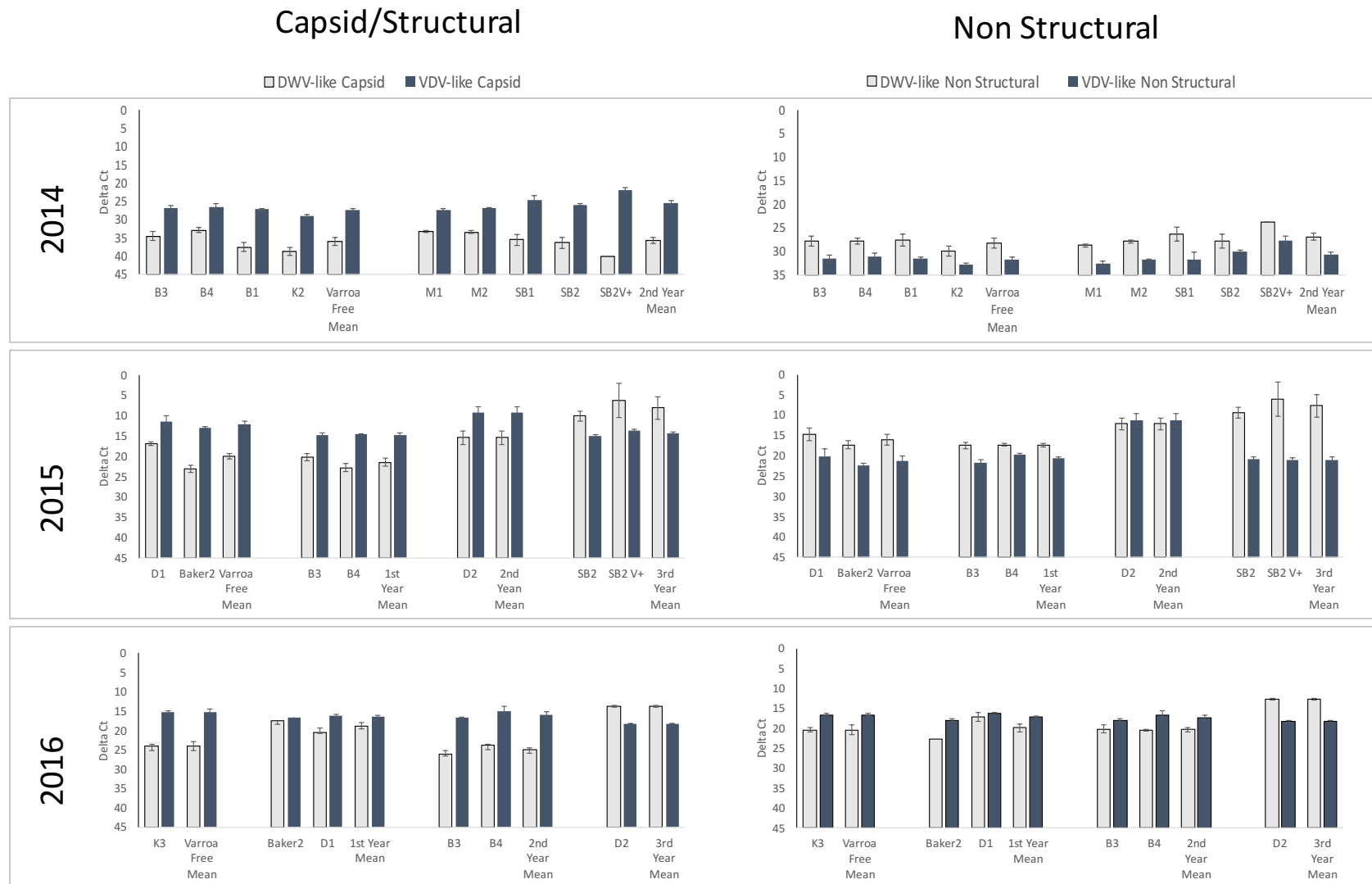


Figure 4.4 Strain specific qPCR results for sampled colonies in 2014, 2015 and 2016. Results displayed as mean delta Ct scores, normalised to honey bee actin levels per sample. For each sampling year, results are separated to show relative levels of DWV-like (pale grey) and VDV-like (dark grey) capsid/structural sequences, and non structural sequences. Colonies sampled each year are arranged from left to right along the x axis according to the length of time that they have been infested with Varroa.

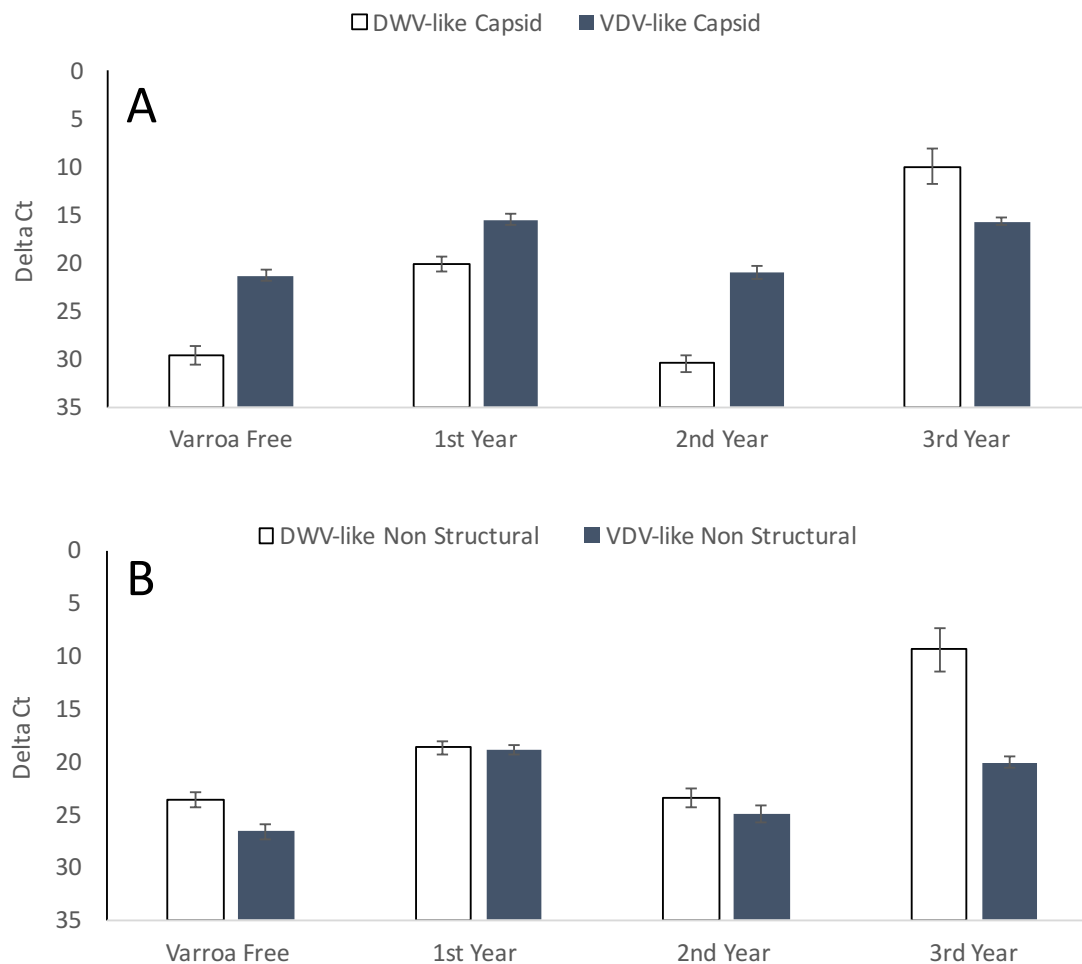


Figure 4.5 DWV and VDV-like capsid (A) and non-structural (B) sequences, mean delta Ct scores for number of years of known Varroa infestation for the sampled apiary

Strain specific qPCR can be used to identify relative levels of DWV-like and VDV-like capsid (structural) and polymerase-coding regions (non-structural). This method does not give exact quantification of the strains present in a sample – but it does give an excellent ‘snap shot’ and allows us to build up a good picture of changes in viral strains over time.

Levels of DWV-like or VDV-like capsid and polymerase sequences were determined for 8 pupae per colony, sampled in the September of 3 sequential years (2014 to 2016) (Fig 4.4). In 2014 all of the colonies sampled from the Ardnamurchan area appear to have a similar, low level virus infection, with predominantly VDV-like capsid coding regions and DWV-like polymerase coding regions. The colonies sampled in 2014 were either *Varroa* free (B3, B4, B1, K2) or had been exposed to *Varroa* parasitism for 2 years (M1, M2, SB1, SB2), however, *Varroa* numbers were very low as the beekeepers had been treating intensively to prevent the mites becoming established in the area. The picture is different by September 2015, with one of the colonies sampled (SB2) having notably higher *Varroa* levels (personal correspondence, Fiona Highet SASA and Kate Atchley (local beekeeper), having been infested with the mites for 3 seasons. All other apiaries sampled continued to have predominantly VDV-like capsid coding regions and DWV-like polymerase coding regions, but samples from SB2 had significantly higher levels of DWV-like polymerase sequences, a notable shift from the same apiary in the previous year ($t(15)=2.455$, $p=0.22$) and from the other Ardnamurchan apiaries in 2015 ($t(55)=7.874$, $p<0.001$). In the non-structural coding region, the picture for the colony SB2 was also notably different to the other sampled colonies, with a greater difference between the levels of DWV-like and VDV-like polymerase coding regions in samples from SB2, indicating an increase in the levels of DWV-like non-coding regions of the virus. The heavily infected colony SB2 was then lost during the winter of 2015/16. In 2016 we again see fairly uniform levels of DWV-like and VDV-like viruses in all apiaries, with the exception of D2, where *Varroa* had been present for 3 years and were becoming established. Again, there appears to be a shift in the predominant capsid coding region present in the apiary, with more DWV-like than VDV-like sequences present. There also appears to be an increase in the DWV-like polymerase coding regions. This pattern is similar to that observed in the most heavily *Varroa* infected colony from the previous year.

The picture is clearer when we look at the mean delta ct results for the Ardnamurchan samples as a product of the number of years for which *Varroa* have been present in the apiary, rather than solely separating samples by the year in which they were collected (Fig

4.5). In *Varroa* free apiaries, and those in their 1st and second years of infestation VDV-like capsid sequences dominate – in *Varroa* free apiaries VDV-like capsid sequences are significantly higher than DWV-like ones ($t(96)=12.244$, $p<0.001$), and the same is true in apiaries in which *Varroa* have been reported for one season ($t(43)=9.485$, $p<0.001$), and two seasons ($t(102)=9.418$, $p<0.001$). In apiaries for which *Varroa* have been present for three seasons the opposite is true, with DWV-like capsid sequences dominating ($t(40)=4.557$, $p<0.001$). For the non-structural coding region DWV-like and VDV-like strains are present at very similar levels in *Varroa* free, year one and year two apiaries, but in apiaries where *Varroa* have been present for 3 years DWV-like sequences dominate ($t(42)=7.929$, $p<0.001$).

4.3.1.1.2. Sequencing of cloned PCR fragments from DWV central region, validation of qPCR

Nested PCR of the central region of DWV was conducted for samples from Ardnamurchan from 2015 to allow validation of the strain specific qPCR results. Two colonies were selected for this procedure – Baker2 (*Varroa* free) and SB1 (*Varroa* positive), with 9 clones being sequenced from 3 pupae from each colony. The FASTA sequences are included in the appendix (Appendix 2). The numbers and proportions of DWV-like, VDV-like and VDV/DWV-like recombinant clones from each colony are shown in Figure 4.6.

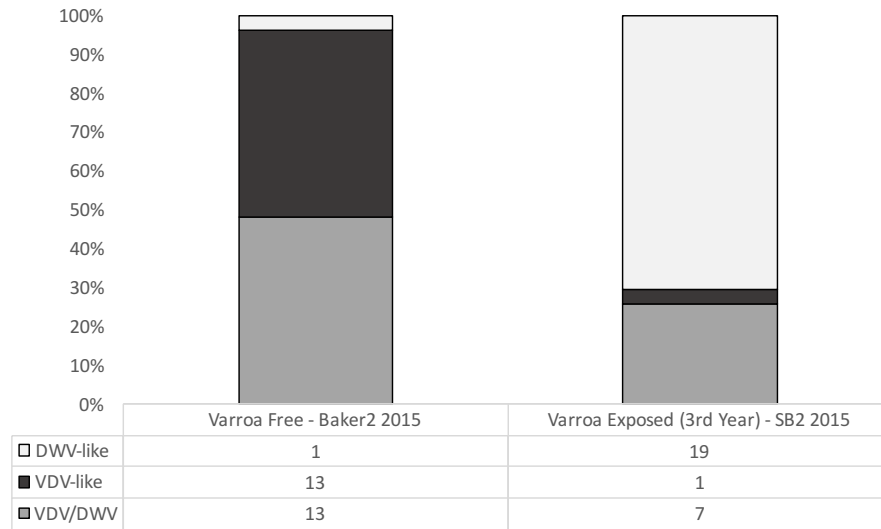


Figure 4.6 Proportions of DWV-like, VDV-like and VDV/DWV-like sequences from a nested PCR of the central region of the virus. Two colonies were selected for this confirmation cloning, Baker2-2015 (*Varroa* free) and SB2-2015 (High *Varroa*). 27 clones from each colony were sequenced (9 clones from each of three individual pupae), and the sequences were assessed as being either DWV-like, VDV-like or recombinants by BLAST search results and CLUSTAL alignment.

The cloned and sequenced PCR products show a similar picture to the qPCR results. Sequencing of the central region of the virus - through regions corresponding to positions 4926 to 6255 of the DWV genome (GenBank Accession Number AJ489744), where recombination has been shown to occur (Ryabov, *et al.*, 2014) - allows us to build up a picture of the strains of the virus that are most prevalent within a colony. In the *Varroa* free colony (Baker2), there is an even split between VDV-like viruses and recombinant strains, with VDV-like structural regions and DWV-like non-structural regions. In the *Varroa* infected colony, SB2, the picture is different, with the majority of amplified, cloned viral sequences being DWV-like and a smaller proportion of recombinant strains. The fact that the sequenced clones from these two colonies show a similar distribution of viral strains to those predicted by the strain specific qPCR assay validates the results of the qPCR assay, giving further evidence for a shift in the predominant DWV-like viral strain as *Varroa* become established in a colony.

Previous studies have identified a narrowing in the diversity of DWV-like viruses that accompanies this shift in the predominant viral strain (Martin, *et al.*, 2012; Ryabov, *et al.*, 2014). To achieve an accurate picture of the diversity of viruses within an individual or population deeper, next generation sequencing (NGS) is necessary. The techniques I have used in this work allow us to confidently analyse the proportions of the main viral strains at certain known points in the viral genome (qPCR), or to take a snapshot of the viruses present within a sample, working on the assumption that the most prevalent viral strains will be the most commonly represented in the amplified, cloned and sequenced PCR products. Alignment of the thousands of sequence fragments generated by Illumina NGS to reference sequences is the most commonly used technique to allow a confident assessment of viral diversity in a sample, but unfortunately this process is expensive and therefore remains beyond the scope of this PhD project. Some research groups have used a ‘high resolution melt’ technique to visualise and quantify the diversity of DWV like viruses in samples (Martin, *et al.*, 2012; Mordecai, *et al.*, 2016a), however neither the published protocol or one conducted with modified primers was able to reliably detect any change in viral diversity in the samples from Ardnamurchan. Results of work done to investigate this method are given in Appendix 3.

4.3.2 Single Apiary Experiment, analysis of the change in predominant strain of DWV in a single colony as *Varroa* infestation takes hold

The sampling of the two experimental colonies maintained at the University of Warwick revealed significantly different trends in *Varroa* mite population levels, as shown in figure 4.7 ($t(24)=3.082$, $p=0.005$).

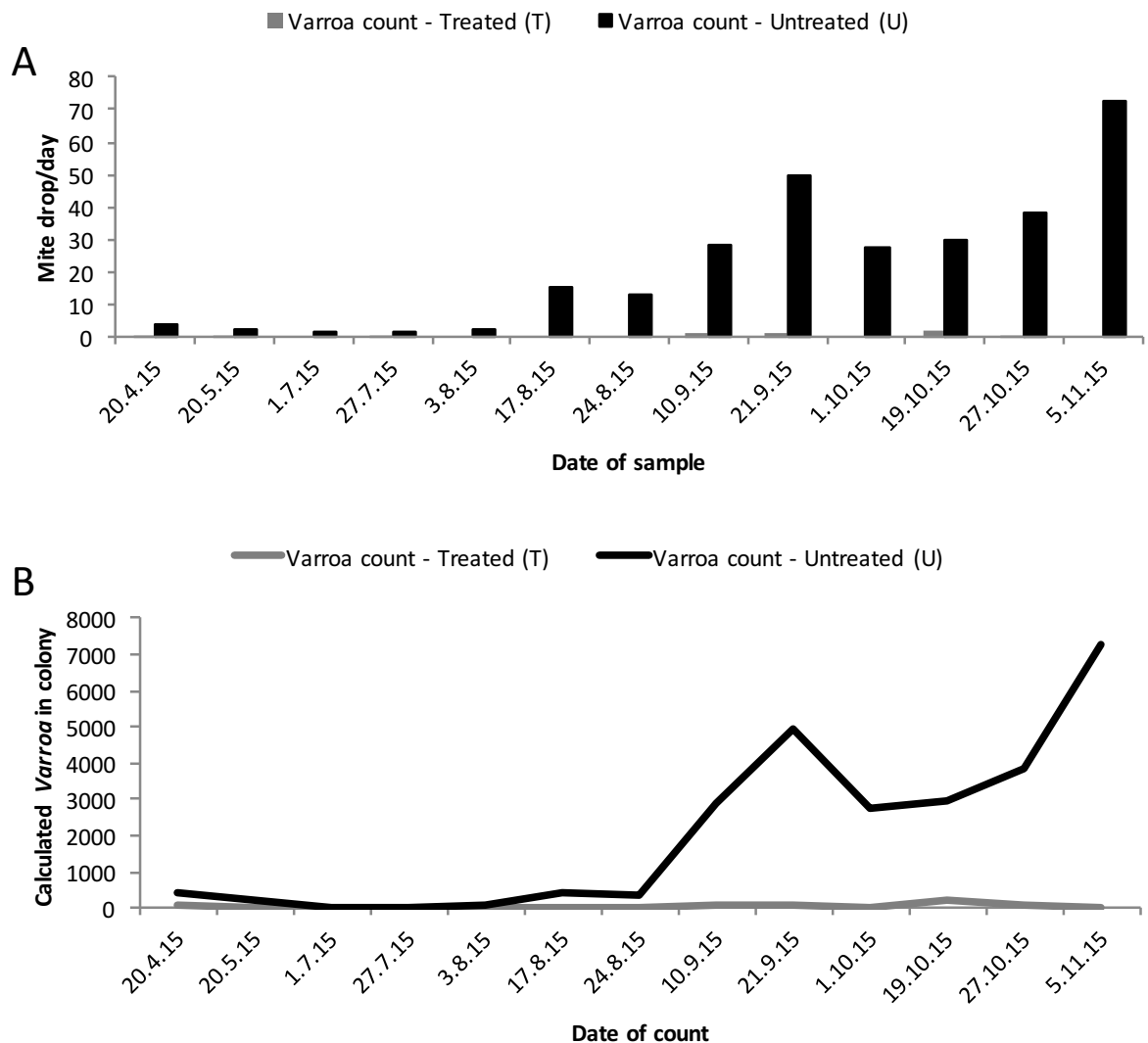


Figure 4.7 *Varroa* mite numbers in the two experimental colonies for the sampling season 2015. Mite numbers were calculated using Natural Mite Mortality method outlined by The National Bee Unit. Figure A shows the mean daily mite drop numbers for each sampling period. Figure B shows the calculated estimate for the total mites per colony at each sampling point.

The levels of *Varroa* remain low in both colonies throughout the spring and early summer, with the mite drops consistently staying at fewer than 10 mites per day in the April to July sampling periods. From August onwards, the *Varroa* numbers in the untreated (U) colony begin to rise dramatically, whilst in the treated (T) colony they remain at fewer than 10 mites/day. This is reflected in the calculated estimates for numbers of mites per colony, with the estimated total mites in the T never rising above 300, in contrast to U where there

were estimated to be 460 total mites in mid-August, raising rapidly to 4967 by late September and up to 7300 by the final sampling point in November.

Samples of bees were collected throughout the season, with the aim of revealing how this unchecked *Varroa* infestation in U altered the level and population of DWV within the colony. For clarity, we will compare the DWV levels and predominant strains from bees collected at the start (20.4.15 – hereafter ‘early’) and end (5.11.15 – hereafter ‘late’) of the season (Figure 4.8).

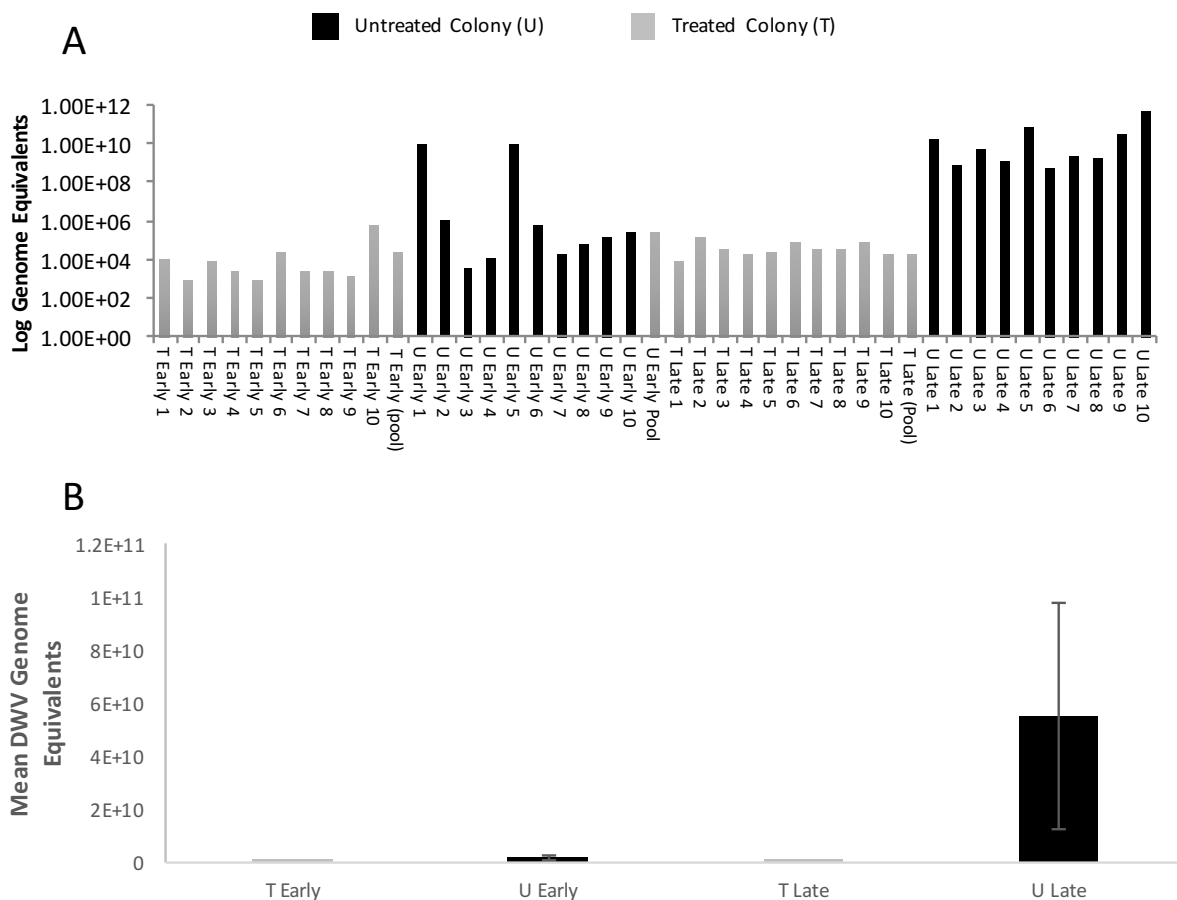


Figure 4.8 DWV levels in adult honey bees from both experimental colonies at the start and end of the sampling season. A. Log₁₀ DWV genome equivalents per individual, B. Mean DWV genome equivalents per sampling group. As hypothesised, the levels of DWV increase dramatically in the U colony whilst remaining the same in colony T.

As found by other research groups (Martin, *et al.*, 2010; Francis, *et al.*, 2013; Locke, *et al.*, 2017), the unchecked expansion in the *Varroa* population within a honey bee colony was reflected in the levels of DWV infection in the adult foraging bees. The untreated (U) colony in this study showed a 30 fold increase in the mean DWV genome number per adult sampled from the start to the end of the season, whereas the DWV levels in the treated (T) colony remained unchanged (Figure 4.8).

When the mean DWV levels are plotted for all of the sampling points across the season (Figure 4.9 B), it appears that the increase in mean DWV level in adult bees follows a similar pattern to that observed in the estimated *Varroa* infestation numbers. The large increase in the estimated *Varroa* population in colony U in mid September (sampling point 21.9.15) was followed by a corresponding increase in the mean DWV level per adult honey bee sampled in early October (1.10.15).

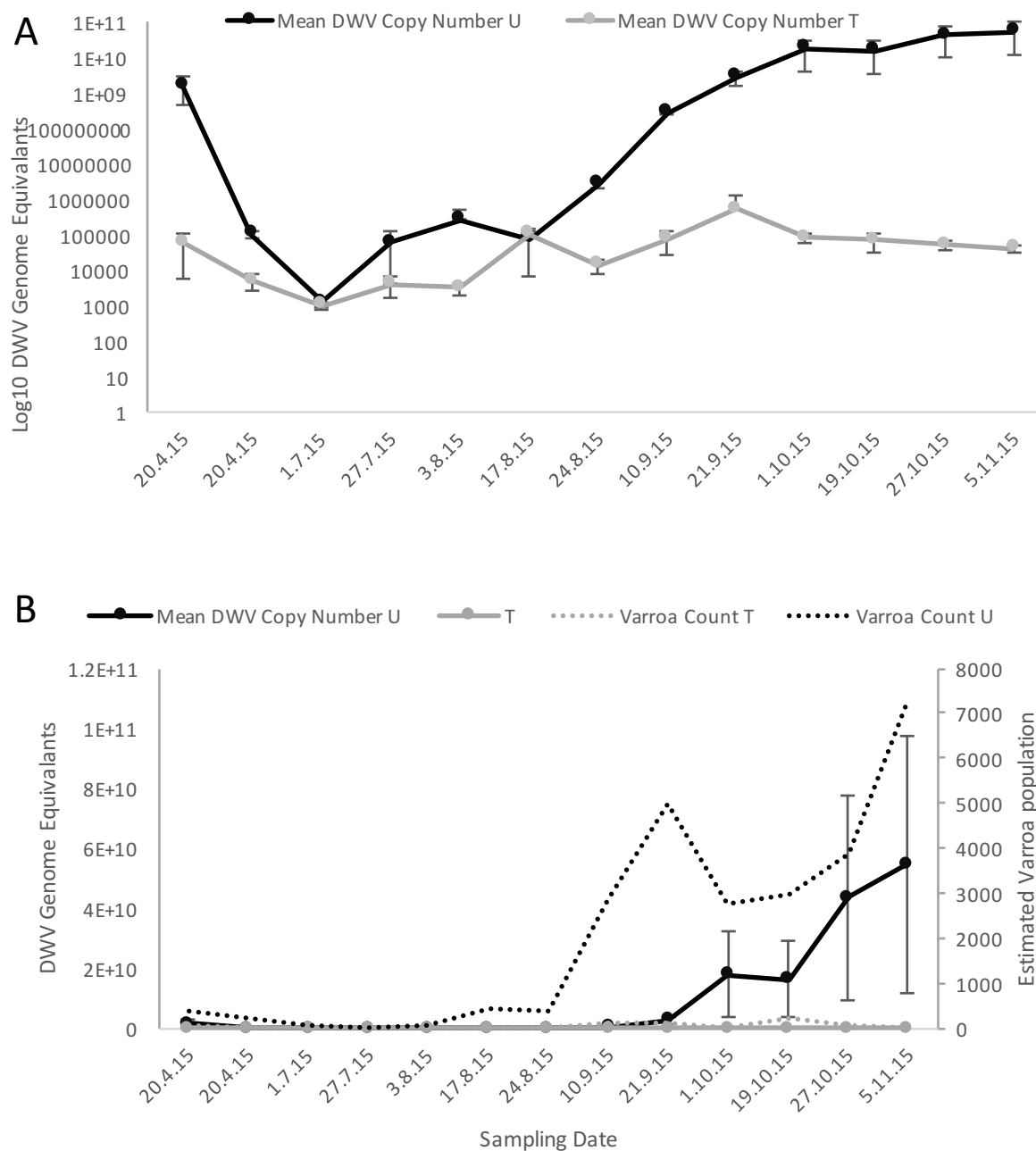


Figure 4.9 *A* Mean DWV genome equivalents per adult honey bee throughout the sampling season. DWV copy number per adult honey bee remains relatively low (at sub clinical levels (Locke, et al., 2017)) in both colonies until September, where the levels in the U colony increase dramatically, whilst the levels in the T colony remain low. Genome equivalents shown using a Log10 scale. *B*. DWV copy number shown on the left axis without the Log10 conversion, Varroa population on the right axis. The large jump in Varroa population in mid-September is followed by a corresponding increase in DWV levels by early October.

The trends in *Varroa* population and DWV levels within the experimental colonies followed those described in the literature (Martin, *et al.*, 2010; Francis, *et al.*, 2013; Locke, *et al.*, 2017), although there were two individuals with ‘high’ DWV levels in the first sample collected from the U colony (figure 4.8 A). These individuals may have been overwintered workers, resuming foraging duties following the renewal of brood rearing in the late winter/early spring ((Seeley and Visscher, 1985; Döke, *et al.*, 2015). The fact that 20% of adult workers have high levels of DWV so early in the season may indicate that the colony suffered from a heavy *Varroa* infestation and corresponding high viral load in the season before the experiment (Locke, *et al.*, 2017).

Having established that the lack of *Varroa* control treatments in colony U lead to a sharp rise in the DWV levels within the colony, it was then possible to test the predominant viral stain within the colony at the beginning and end of the experiment. This was intended to provide more information on whether high levels of *Varroa* mediated transmission of the DWV virus alters the predominant strain of the virus within a colony.

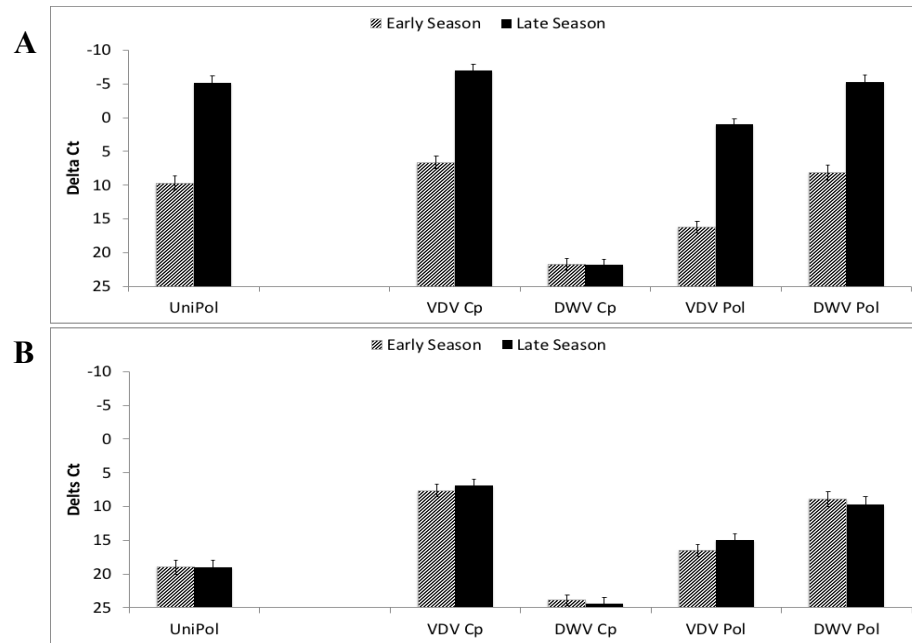


Figure 4.10 Strain specific qPCR results for early (hashed bars) and late (black bars) season samples from colony U (A) and colony T (B). Universal Polymerase primers (UniPol) detect the same dramatic increase in DWV levels shown in figures 4.8 and 4.9. Strain specific primers show that there was a corresponding increase in VDV type capsid sequences (VDV Cp), and in VDV and DWV type polymerase sequences (VDV Pol, DWV Pol).

Figure 4.10 (A) shows the results of the strain specific qPCR on early season and late season honey bees from colony U. The DWV virus present at low levels at the start of the season had predominantly VDV like capsid sequences and VDV like or DWV like polymerase sequences. The samples collected at the end of the season had higher levels of virus, but the proportions of DVW and VDV like sequences remained the same. This would indicate that, whilst *Varroa* infestation had a large effect on the levels of DWV, it did not significantly alter the mixture of strains of virus within this colony. In the treated colony (T) the pattern was very similar to the one shown in the untreated bees (Figure 4.10 B), qPCR revealed the mix of DWV-like and VDV-like capsid and polymerase sequences to be the very similar to those found in the untreated colony at both the start and end of the season.

4.4 Discussion

4.4.1. Analysis of predominant strain of DWV in the Ardnamurchan Peninsula, an area at the forefront of *Varroa* expansion in the UK

The analysis of the honey bee pupae samples collected from the isolated Peninsula of Ardnamurchan over a three-year period gives a valuable insight into how the introduction of *Varroa* changes the virus population within an area. DWV levels remained low throughout the sampling period, a testament to the success of the Ardnamurchan beekeepers intensive *Varroa* monitoring and treatment regime. However, in spite of this intensive work, the mites were able to become more established in two of the experimental apiaries (in each case in the third year of their presence) and in bees from these colonies we saw a marked alteration in the predominant strain of DWV. In the Ardnamurchan samples we observed a shift from a mixed population of VDV-like and recombinant (with VDV-like structural regions and DWV-like capsid sequences) viruses in the *Varroa* free and ‘early stage’ *Varroa* exposed colonies, towards a predominantly DWV-like virus population in the colonies with a more established *Varroa* infestation.

Our previous work (Ryabov, *et al.*, 2014) describes a shift in the predominate DWV strain, alongside a narrowing in the viral diversity when *Varroa* naïve bees are introduced to an infested, high DWV colony. In the 2014 experiment, Scottish bees (in this case from Colonsay rather than Ardnamurchan, but shown to have a similar low level, mixed DWV infection) were introduced to a Warwickshire colony with an established *Varroa* infestation. The individuals exposed to the Warwickshire *Varroa* mostly went on to show high levels of a recombinant strain of DWV/VDV, a strain which dominates the local area, and we concluded that this strain has a selective advantage when transmitted by *Varroa*. However, in the Ardnamurchan samples the full length DWV-like viruses dominate within colonies where virus transmission has become predominately *Varroa* mediated, as opposed to the oral and vertical transmission that maintains the low level mixed population infection in *Varroa* naïve colonies.

The shift from the mixed virus population to a principally DWV-like population upon the introduction of *Varroa* to Ardnamurchan mirrors that reported in Hawaii (Martin, *et al.*, 2012; Brettell, *et al.*, 2017). The experiment described in this chapter emulates the Hawaii study in that it looks at changes in the DWV like viruses in the first few years after *Varroa* introduction. In both experiments DWV-like viruses dominate within a few years, even though in Hawaii very little *Varroa* control was attempted in the sampled colonies (resulting in a rapid increase in DWV titres within the honey bee population) and in Ardnamurchan DWV levels remained low due to intensive *Varroa* control.

I believe that there are two potential explanations for the results of this Ardnamurchan study when considered alongside the mixed conclusions drawn within the published literature with regard to virulent or avirulent strains of DWV. Firstly, it is possible that, instead of a single virulent strain of the virus having a selective advantage when transmitted by *Varroa* (i.e. either a full length DWV like sequence, or a specific DWV/VDV recombinant) there is a more specific, shorter, section of the viral genome that confers this selective advantage. This crucial genetic element could be contained within DWV-like, VDV-like and recombinant strains of the virus, and be present at low levels in the mixed population due to recombination and natural variation. The strain which goes on to dominate following *Varroa* introduction is dependent on the strains present within the bees prior to the arrival of the mite, or alternatively, the strains carried by *Varroa* into an area. Alternatively, it could be that DWV-like strains dominate within colonies where *Varroa* have recently become established (as in Ardnamurchan and Hawaii) but that this is not a final, stable, population and sampling in subsequent years could reveal different ‘dominant’ strains.

To test these theories, it will be necessary to sequence the dominant strain of DWV in all the areas where *Varroa* mites have become established. Alignment of these global full length DWV sequences would allow identification of any conserved elements of the genome, potential candidates for conferring this selective advantage. A reverse genetic system, which is now possible following the identification and publication of an infectious

full length DWV clone (Lamp, *et al.*, 2016), would allow testing of viral clones containing these candidate sequences. Identification of the virulent strains of DWV, or of the element of the DWV genome that confers a selective advantage during *Varroa* transmission, would shed more light on why the DWV/*Varroa* combination has proved so deleterious to honey bee health worldwide. It may also provide potential targets for developing anti-viral treatments.

4.4.2 Single Apiary Experiment, analysis of the change in predominant strain of DWV in a single colony as *Varroa* infestation takes hold

Whilst the unchecked expansion of the *Varroa* population lead to a dramatic increase in the DWV levels within the experimental colony, the comparative ratios of DWV-like viral strains present did not appear to change.

Previous studies have established that the most prevalent strain of DWV-like within *Varroa* exposed bees in our experimental apiaries in Warwickshire is a VDV/DWV recombinant (Moore, *et al.*, 2011; Ryabov, *et al.*, 2014). Our qPCR results for the bees sampled throughout the 2015 season appear to show that this VDV/DWV recombinant strain predominates within the colony, both at the start and end of the season, irrespective of colony *Varroa* numbers and DWV titres within the sampled bees.

The presence of ‘high’ DWV bees in the early season sample, in spite of the low *Varroa* numbers at that point in the season, is a strong indication that the overwinter bees had high levels of DWV as a result of *Varroa* infestation in the previous season (Locke, *et al.*, 2017). This suggests that any alteration on the DWV population due to high *Varroa* load had already occurred within this colony, and the dominant strain persists overwinter, even after *Varroa* treatment has taken place. The results of this experiment reveal that the strain of DWV present within a *Varroa* infested apiary remains stable throughout the season and, apparently, across a number of seasons. Unfortunately, it was not possible to analyse samples from the same colony from subsequent seasons as the high viral load and

unchecked *Varroa* population weakened the colony and it did not survive through the winter.

4.3.3 Techniques for determining DWV strain or diversity within honey bee samples

The techniques used to give a picture of DWV strains within honey bee samples within this chapter – strain specific qPCR and central region cloning and sequencing - only give ‘snapshots’ of the DWV-like viruses present rather than a complete picture of all of the DWV like viruses in a sample. Next generation RNA-sequencing is required to reveal the full extent of all of the DWV RNA sequences present, and this technique has been used successfully by other papers in the literature (Ryabov, *et al.*, 2014; Wood, *et al.*, 2014; Mordecai, *et al.*, 2016a; Mordecai, *et al.*, 2016b). However, this technique is expensive and to analyse the many individual samples that have been collected and tested for the experiments outlined in this chapter was unfortunately not financially possible.

The requirement for an affordable, reliable assay for DWV strain or diversity has been addressed by both our and other research groups, and two methods have been outlined in the published literature (Martin, *et al.*, 2012; Ryabov, *et al.*, 2014; Kevill, *et al.*, 2017) – these are the qPCR followed by validation through central region cloning methods outlined in this chapter and the high resolution melt technique (HRM) first used by Martin *et al.*, (2012) in the Hawaii study. I attempted to use the HRM technique using both the published oligos and alternative ones designed to anneal to the ‘local’ Warwickshire strain of DWV. Unfortunately, I was unable to produce reliable results using the assay, possibly due to the very low levels of virus in the Ardnamurchan samples or due to the lack of genetic variability in the Warwickshire single apiary experiment.

However, the strain specific qPCR assay used in this chapter has been shown to reliably reveal the proportions of DWV-like or VDV-like capsid sequences within a sample, with the qPCR results being validated by Illumina RNA-sequencing in the 2014 Ryabov *et al.*

paper. A similar assay, although with an additional set of oligos to allow the quantification of three DWV variants, has been published by Kevill, *et al.* (2017), and again the results of the assay are validated by next-generation sequence data. I feel that the methods outlined in this chapter represent a reliable, affordable way to analyse DWV genotype mixtures and predominant strains within honey bee samples.

Chapter 5 - *Varroa destructor*, Deformed Wing Virus, and developmental abnormalities in honeybees.

5.1 Introduction.

Overt DWV infections are characterized by visible disease symptoms (deformed wings, bloated and shortened abdomen, discolouring) and are associated within honeybee colonies with *Varroa* feeding, leading to elevated DWV levels within developing pupae. Laboratory infection assays have shown that symptomatic bees result from DWV injection in the absence of *Varroa*, suggesting that DWV is the causative agent of the deformed-wing syndrome (Moeckel, et al., 2011).

The mechanism by which *Varroa* and DWV causes disease in individual bees is not well understood (de Miranda & Genersch, 2010). A priori, a “successful” infection involves failure of the bee immune system to prevent proliferation of the virus, alongside an effect of the virus and / or *Varroa* on the physical development of the bee pupa. The transition from the bee pupa to the adult stage is a highly dynamic, genetically programmed process that entails remodelling of tissues and growth of new organs (Soares, *et al.*, 2013; Belles and Santos, 2014; Vleurinck, *et al.*, 2016). It is possible that DWV infection of honeybee larvae, prepupae and pupae disturbs development to the adult through effects on the expression of genes associated with developmental processes. This is known to happen in other systems: for example, infection with the baculovirus *Lymantria dispar* nuclear polyhedrosis virus affects the development of its insect hosts *Lymantria dispar* (the gypsy moth) and *Spodoptera frugiperda* (the fall armyworm) by altering the levels of ecdysteroid, the insect moulting hormone, in the haemolymph of infected individuals (Burand and Park, 1992; Park, *et al.*, 1993; Park, *et al.*, 1996). Ecdysteroid is well studied as a moulting hormone in insects, and it has been established to play a critical role in the regulation of programmed development (Truman and Riddiford, 2002).

Exposure to pathogens, including DWV, has been shown to induce wide scale transcriptome changes in the host honeybee (Ryabov, *et al.*, 2014; Doublet, *et al.*, 2017), however the published works on this subject have produced conflicting results – for example Ryabov *et al.* (2014) reported that *Varroa*-exposed, DWV infected pupae had 951 significantly differentially expressed (DE) genes (approximately 9% of the honeybee genome) when contrasted with control individuals. Gene ontology analysis of these DE genes did not reveal any coordinated response to the pathogen load, with no significantly over represented gene ontology biological process terms. This suggested that unchecked DWV replication lead to dysregulation of the usual transcriptional pattern within honeybee cells or mRNA stability, as has been previously reported in picornavirus infection of mammalian cells (Grinde, *et al.*, 2007; Doukas and Sarnow, 2011; Rozovics, *et al.*, 2012). However, Doublet *et al.*, (2017) performed a meta analysis of multiple transcriptome analysis experiments and revealed that the transcriptional response to *Varroa*/virus infection was characterised by the DE of genes from two immune pathways – Imd (*iap2* and *rel* genes) and Toll pathways (*tube* and *def-2* genes). Notably, neither of the studies mentioned above have detected any DE of genes associated with honeybee development. It is possible that fluctuations in the expression of developmental genes or hormonal receptors are occurring but not being detected due to the methodologies of the studies in the current literature. Ecdysone receptors (Mello, *et al.*, 2014) and homeobox developmental genes (Walldorf, *et al.*, 2000a) have been shown to have transient expression in developing honeybees, tightly linked to developmental stage. As a consequence of this, any experimental design involving pooled samples or only sampling at a single developmental stage or time point may not detect any perturbation to the expression pattern of developmental genes.

The results of a recent microarray analysis of *Varroa*-parasitised bee pupae collected from a naturally infested colony maintained at Rothamsted Research UK may reveal more about how elevated DWV infection in honeybee pupae causes developmental abnormalities (D. Chandler, unpublished data). Insect morphological development is

governed by a suite of evolutionarily conserved homeodomain proteins, transcription factors and co factors which regulate cell fate and cell type gene expression to determine insect segment morphology and system development. (Ladam and Sagerstrom, 2014; Walldorf, et al., 2000). The microarray data indicated that *Varroa* parasitism (and therefore viral load, as DWV infection was shown to be inextricably linked to *Varroa* parasitism in the sampled bees) had a strong effect on the expression of these homeobox genes, with 70% differentially expressed in response to *Varroa* mite burden. This is a novel result, which has not been observed by other research groups; the work described in this thesis further explores this phenomena, revealing more about how universal and reliable this disturbance of developmental gene expression in response to DWV infection and *Varroa* feeding is in honeybee pupae.

5.1.2 Insect developmental genes

Holometabolous insects, such as honey bees, undergo complete metamorphosis during their development, transitioning through egg, larval, pupal and adult life stages (Truman and Riddiford, 2002; Kayukawa, et al., 2017). This complex process is governed by a pattern of gene expression which has been most extensively studied in the model organism, *Drosophila melanogaster* (Arbeitman, et al., 2002). The majority of published work focuses on *D. melanogaster* embryogenesis, with less focus on the pupal to adult metamorphosis that appears to be affected by DWV infection in honey bees.

The major morphological transitions that occur during larval-to-adult insect metamorphosis are initiated by changes in the titre of the hormone 20-hydroxyecdysone (20-HE). This operates through an ecdysone receptor protein complex (ECR-C) formed with the ecdysone receptor (ECR) and its heterodimeric partner ultraspiracle (USP) (Barchuk, et al., 2008; Gauhar, et al., 2009). The ECR-C modulates the expression of a group of 20-HE responding genes that encode transcription factors (Gauhar, et al., 2009) as well as controlling the length of the pupal development period (Barchuk, et al., 2008). Insect morphological development is also regulated by a suite of evolutionary conserved homeodomain proteins: these transcription factors, in combination with cofactors,

regulate cell fate and cell-type gene expression to determine insect segment morphology and the development of appendages, organs, the nervous system and the neuroendocrine systems (Hayashi and Scott, 1990; Ladam and Sagerstrom, 2014). Most of the research on insect homeobox gene function has focused on embryonic development (mainly in *Drosophila*) (Deng, *et al.*, 2012) but it has been shown recently that they also have critical developmental functions during larval-to-adult metamorphosis (Deng, *et al.*, 2012). The ecdysone and homeodomain protein signalling pathways co-regulate morphological development (Soares, *et al.*, 2013), although the precise details of this interaction are not well understood.

As mentioned above, insect homeobox (or Hox) gene functions have been studied most extensively in *Drosophila*; however honey bee homologs of known *Drosophila* homeobox-containing genes have been identified (Walldorf, *et al.*, 1989; Zhong, *et al.*, 2008; Zhong and Holland, 2011) and their expression patterns described (Walldorf, *et al.*, 2000b). Similarly, the role of juvenile hormone (JH) and the mechanism of action of ecdysteroids in insects were initially studied in *D. melanogaster* molting and metamorphosis (Belles and Santos, 2014). These ecdysteroids have now been shown to be crucial in caste determination in eusocial insects, such as the bumblebee *Bombus terrestris* (Geva, *et al.*, 2005) and in the honey bee (Hartfelder and Engels, 1998), playing a role in the regulation of differential morphogenesis alongside JH (Mello, *et al.*, 2014).

In order to establish how these crucial developmental pathways are disturbed by *Varroa* feeding and DWV infection, a series of natural and experimental infection experiments were carried out. The results reveal more about how DWV affects developing honey bee pupae, possibly pointing towards an explanation for the symptoms associated with DWV infection. Interestingly, we also show unexpected between-colony variation in the developmental response to DWV, a result which may shed light on why previous studies have not detected any significant perturbation to these gene pathways during DWV infection.

The aim of this work was to test the hypothesis that DWV infection during crucial stages of honey bee pupation and metamorphosis causes disturbances to the expression of developmental gene pathways, including ecdysone receptor signalling and homeobox developmental genes, leading to the characteristic DWV symptoms. This hypothesis is tested through comprehensive measuring of the expression of key developmental genes in honey bee pupae infected with DWV, both via *Varroa* feeding and experimental inoculation.

5.2 Materials and Methods

5.2.1 Analysis of developmental gene expression in naturally *Varroa* infested honey bee pupae

To further explore the observational microarray profiling results described in the introduction to this chapter, a sample of honey bee pupae were collected from an independent apiary in a different part of the UK to establish if the disturbance to the Hox genes was detectable in an independent sample.

Worker honey bee pupae were collected from a single, heavily *Varroa*-infested *Apis mellifera* colony (Colony ID FRANK79) from an apiary maintained by the National Bee Unit, the Animal and Plant Health Agency (APHA), Sand Hutton, York. The colony was recorded as having a naturally mated queen, and was thought to represent a genetic mixture of European subspecies. Pupae were removed from two frames of capped brood during a single day of sampling (1/9/2016). The age of each pupa was determined using the standardised larval and pupal ageing chart (Methods Chapter, Figure 2.4) and the number of adult and juvenile *Varroa* mites in the cell with each pupae was also recorded. The bees were then flash frozen in liquid nitrogen prior to storage at minus 80 °C. A total of 150 individual pupae were collected and categorized, the age and *Varroa* load distribution of pupae is shown in Figure 5.1.

RNA extraction and clean up was carried out according to the methods in Chapter 2 of this thesis (Section 2.6).

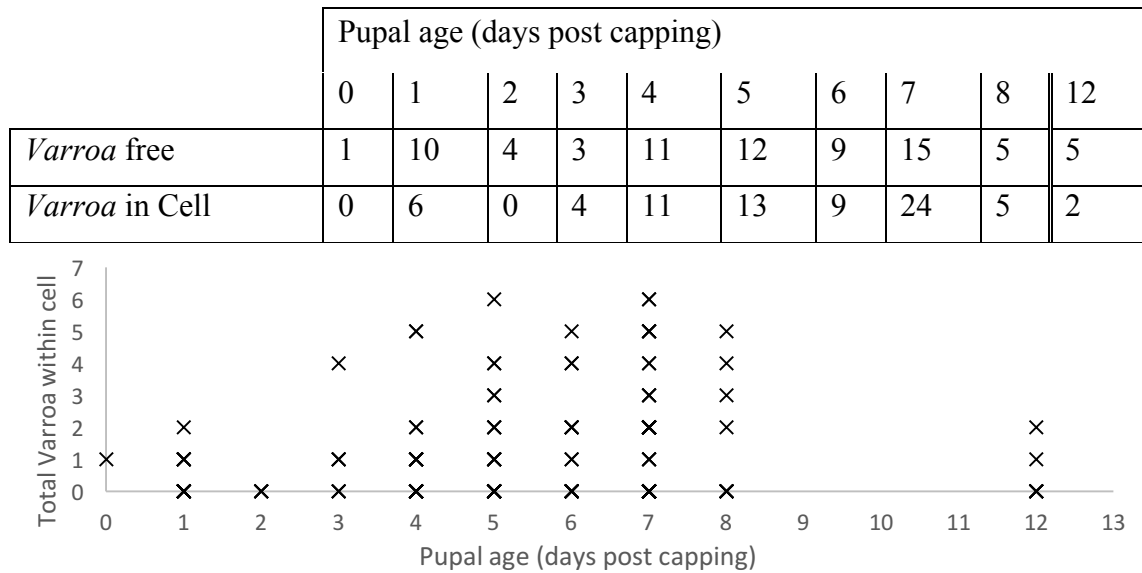


Figure 5.1. Sample numbers and age/*Varroa* distribution in sample from National Bee Unit, The Animal and Plant Health Agency (APHA), Sand Hutton, York.

DWV copy number was determined for each individual pupae using qPCR (as in Chapter 2, Section 2.7.2). This allowed each individual to be categorized by age (in days post capping) and as having ‘high’ or ‘low’ DWV levels. (As discussed in Chapter 3 of this work, it has been established in the literature that symptomatic individuals typically have a viral load of over 10^9 copies of DWV per individual, and that the infection is typically bimodal, with a clear distinction between ‘infected’ individuals with viral loads of around 10^9 or above, and asymptomatic individuals with significantly lower viral loads (Zioni, *et al.*, 2011; Ryabov, *et al.*, 2014). This allowed us to categorise any pupae with a load of over 10^9 viral copies as being ‘high virus’.)

Once we had information on the age, *Varroa* load and DWV levels of each sampled pupae, a subset of individuals was chosen for further analysis. Due to the age distribution of samples, they were split into 6 age classes; Pre-pupae (with individuals up to 3 days post capping), 4 days post capping, 5 days post capping, 6 days post capping, 7 days post capping and 8+ days post capping. For each age class, 6 to 9 *Varroa* free and 6 to 9 *Varroa* exposed individuals were selected for this further analysis. The objective of this observational experiment was to test for any perturbation to developmental gene

expression, either in the Hox genes or genes from the ecdysone receptor pathway. Primer sets were designed to anneal to regions of the following genes:

Table 5.1 Developmental genes selected for quantification

	Name	Symbo l	Class	Chromosome/ Location	NCBI Gene ID	BeeBase ID	Function in insects (according to Flybase)
Hox	Distalless	Dll	ANTP/Dlx	LG13 NC_007082.3	726710	BEEBASE: GB40095	Expression is focused in limb primordia. Dll specifies limb location (distal) versus body wall (proximal).
	Extradenticle	Exd	TALE/Pbx	LG3 NC_007072.3	408763	BEEBASE: GB51904	Nuclear transcription factor, involved in in antennal development, proximal limb and wing patterning and the development of the undifferentiated eye field.
	Buttonless	Btn	ANTP/Meox	LG7 NC_007076.3	725407	BEEBASE: GB47954	Expressed in dorsal median cells, and the development of the insect nervous system
	Abdominal A	Abd-A	ANTP/Hox6-8	LG16 NC_007085.3	410643	BEEBASE: GB51287	Component of the bithorax complex, controlling the fate of developing embryonic segments
	Extra extra	Exex	ANTP/Mnx	LG13 NC_007082.3	726370	BEEBASE: GB54763	Controls the differentiation of developing motor neurons and the projection of axons to ventral body wall muscles.
	Drop1	Dr1	ANTP/Msx	LG1 NC_007070.3	724456	BEEBASE: GB47493	Involved in the patterning of the wing disk and in the development of muscle, neuron and glial cells.
EcR	Apis mellifera ecdysone receptor (Ecr), transcript variant A	EcR-A		LG8 NC_007077.3	406084	BEEBASE: GB48059	Binds to the hormone 20-Hydroxyecdysone during insect development leading to a major activation cascade of genes involved in molting, neural diversity and temporal patterning.
	Apis mellifera ecdysone receptor (Ecr), transcript variant B	EcR-B		LG8 NC_007077.3	406084	BEEBASE: GB48059	Binds to the hormone 20-Hydroxyecdysone during insect development leading to a major activation cascade of genes involved in molting, neural diversity and temporal patterning.
	Insulin-like peptide receptor	InR		LG9 NC_007078.3	411297	BEEBASE: GB53353	Involved in insulin binding, protein binding and hormonal control of development. Involved in the response to 20-hydroxyecdysone
	Crooked legs	Crol		LG11 NC_037648.1	409470	BEEBASE: GB54040	Zinc finger transcription factor induced by 20-hydroxyecdysone at the onset of metamorphosis. Involved in wing and eye development.

Genes were selected because of a). level of up or down regulation in original microarray experiment, and b). the function or time of expression in drosophila.

Primer sets were designed to anneal to and amplify reverse transcribed mRNA sequences for the selected genes. These primers were tested in qPCR conditions for single product melt curves, and the amplicons were run on a gel and then sent for sequencing to check that the oligos were annealing only to the desired cDNA transcripts. Once that it had been established that the primer sets were working as required, qPCR was carried out on the selected honey bee samples, as described in Methods chapter (2.6.2.) A full list of primers can be found in the appendix of this work.

5.2.2 Analysis of developmental gene expression in experimentally DWV injected honey bee pupae

The expression of developmental genes in honey bees is known to be transient and tightly linked to developmental stage (Walldorf, *et al.*, 2000b; Mello, *et al.*, 2014). In order to accurately quantify changes in the expression patterns of these genes in response to DWV infection, an experimental system was designed which allowed controlled dose applications of the virus in age-matched pupae.

A set of white eyed pupae (4 days post capping, Figure 2.4) from a low *Varroa* colony maintained at the apiary at the Warwick Crop Centre, Wellesbourne, were collected on 19.7.2016 and injected as described in section 2.4.1. A dose of DWV that had previously been established to lead to full infection in the majority of treated individuals was selected, each pupae was injected with 1×10^{-5} ng viral RNA. Control pupae (from the same colony, collected on the same day, all of the same developmental stage) were injected with PBS alone. Six individual pupae were sampled for each treatment (DWV injected or sham injected) at the following time points:

1 hours post injection (h.p.i), 10 h.p.i, 24 h.p.i, 33 h.p.i, 48 h.p.i, 58 h.p.i, 72 h.p.i, 80 h.p.i, 96 h.p.i and 105 h.p.i. (see Figure 5.2)

Samples collected were flash frozen in liquid nitrogen and stored at -80°C. RNA was extracted and processed, and qPCR carried out as described in section 2.6 of this work.

The full experiment was then repeated on 8.5.2017 with individuals from a different honey bee colony from the same apiary to allow us to compare the responses in genetically distinct bees. The sampling times for the second injection experiment were as follows:

1 hours post injection (h.p.i), 10 h.p.i, 24 h.p.i, 33 h.p.i, 48 h.p.i, 58 h.p.i, 72 h.p.i, 80 h.p.i. (see Figure 5.2)

These experiments are referred to as ‘Experiment 1’ and ‘Experiment 2’ in Results section 5.3.2

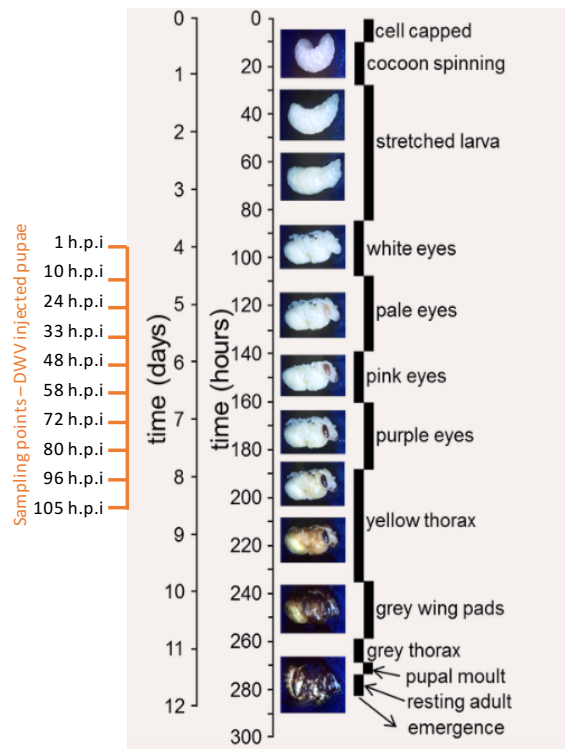


Figure 5.2. Sampling points for analysis of developmental gene expression in experimentally DWV injected honey bee pupae shown in relation to days post capping and developmental stage of pupae. Experiment 1, all sampling points collected. Experiment 2, modified sampling following analysis of experiment 1, samples collected up to 80 h.p.i.

5.2.3 Multi colony injection experiment

In order to further investigate the different patterns in developmental gene expression observed in genetically distinct honey bee colonies, a large scale injection experiment was carried out. Samples of *Varroa* naïve, white eye pupae (4 days post capping, Figure 2.4) were collected from 6 honey bee colonies on the Wellesbourne site on 17.7.2016. Two of the colonies sampled (T1 and T2) were located in the Townsend apiary (Methods Chapter 2.1) purchased as nucleus colonies, of Buckfast type bees (Österlund, 1983) in the season prior to sampling. The other sampled colonies were located on the Reservoir Site, three (R1, R2 and R3) were well established experimental colonies which had been on the site for a number of years and had been allowed to re-queen naturally when required through swarming, colony division (swarm control) and supersedure. The final sampled colony (RS1) was also located on the reservoir apiary, and was a recently collected swarm of bees from the Coventry area (approximately 15 miles from the apiary). Consequently, little was known about the history of these bees but the colony had become established and the queen was laying well.

A sample of brood was collected from each colony on a single day, and 40 age matched, *Varroa* naïve, white eye pupae were extracted per colony. Each individual was then injected with either a single infectious dose of DWV (1×10^{-5} ng DWV RNA) or with the equal volume of PBS. The injected insects were then stored in petri dishes in a humidity controlled incubator (33°C, 90% humidity). Injected pupae were collected and flash frozen in liquid nitrogen at 24 h (10 individuals per colony) and 96 h post inoculation (10 individuals per colony).

After sample collection, RNA was extracted and processed, and qPCR carried out as described in section 2.6 of this work.

5.3 Results

5.3.1 Analysis of developmental gene expression in naturally *Varroa* infested honey bee pupae

In order to further investigate how developmental gene expression differs in honey bee pupae exposed to *Varroa* mites and DWV virus, when compared to normal gene expression in *Varroa* naïve pupae, an analysis was done of naturally infested honey bees sampled from a colony maintained by the National Bee Unit, APHA, Sand Hutton, York. The age matched pupae were tested for DWV levels (fig 5.2) and then a comparative analysis of the expression levels of a number of Hox and EcR pathway genes in the two groups - Control (*Varroa* free, low DWV) and *Varroa* Exposed (*Varroa* in cell, high DWV).

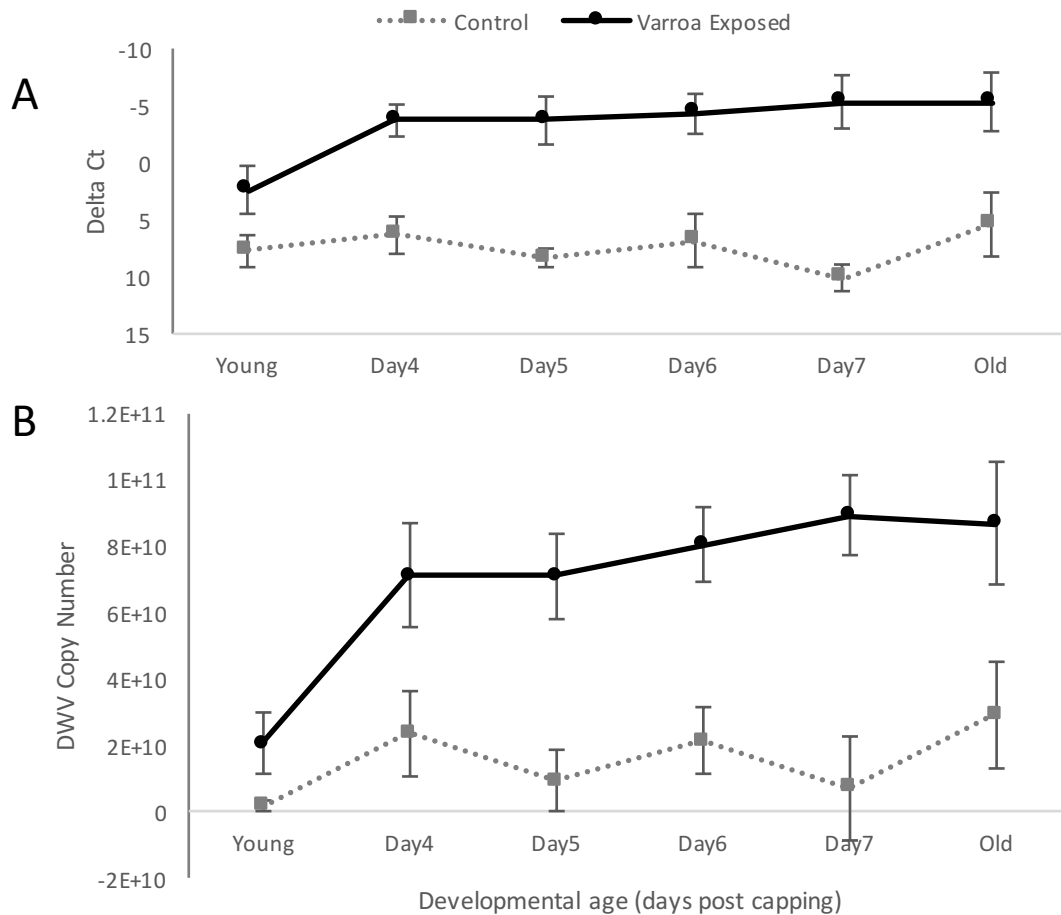


Figure 5.3. DWV levels in two treatment groups of naturally *Varroa* exposed honey bee pupae sampled from a colony maintained by the National Bee Unit, York UK. Points represent the mean value from 6 – 9 individual bees. Each bee was aged using a standardised larval and pupal ageing chart. The ‘young’ age category refers to pre-pupae (with individuals up to 3 days post capping), while ‘old’ refers to 8+ days post capping. qPCR results shown as deltaCt numbers (A) and as calculated DWV copy numbers per individual pupae (B). Error bars are standard error of the mean. By day 4 the sampled pupae had significantly higher levels on DWV if they had been exposed to *Varroa* mites $t(15)=5.319, p<0.001$.

As expected, the DWV levels in the *Varroa* exposed pupae were higher than those found in the *Varroa* naïve individuals (by 4 days post capping (Day4) the difference was significant $t(15)=5.319, p<0.001$.) The increase in virus titre is greatest between the ‘young’ pupae and those sampled at 4 days post capping. This rapid accumulation of DWV genomes within the first 48h of exposure via *Varroa* feeding is similar to the pattern of DWV infection described in experimentally inoculated bees described in Chapter 3 of this work. During this time period (0 to 3 days post capping) the honey bees are

undergoing extensive metamorphosis, known as the prepupal stage with the pupae developing within the larval skin. At 4 days post capping the bees undergo the fifth moult, when the external features of the adult honey bee become visible, although the majority of the internal organs are yet to form (Stell, 2012). It is during this period of honey bee development that the accumulation of DWV appears to be at its peak in *Varroa* exposed bees.

These samples were then used for further qPCR experiments to establish any disturbances to the normal expression levels of Hox genes (Figure 5.4).

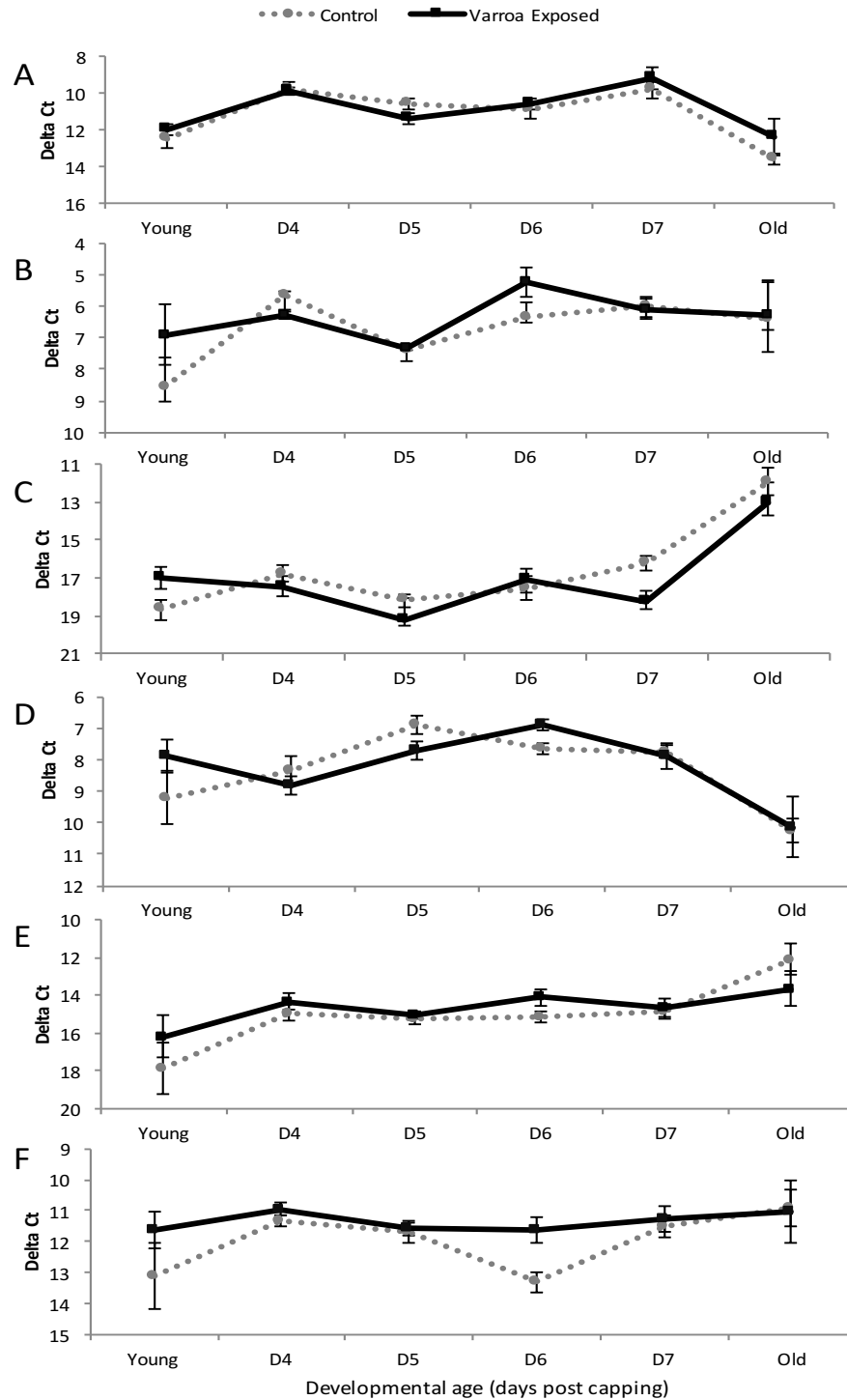


Figure 5.4. qPCR results, revealing the pattern of gene expression of 6 Hox genes in two treatment groups of naturally Varroa exposed honey bee pupae sampled from a colony maintained by the National Bee Unit, York UK. Points represent the mean value from 6 – 9 individual bees. Each bee was aged using a standardised larval and pupal ageing chart. The 'young' age category refers to pre-pupae (with individuals up to 3 days post capping), while 'old' refers to 8+ days post capping. qPCR results shown as deltaCt numbers: A. Distalless (Dll). B. Extradenticle (Ext). C. Buttonless (Btn). D. Abdominal A (AbdA). E. ExtraExtra (ExEx). F. Drop1 (Dr1). Mean expression levels, normalized to actin (Delta Ct) are shown for Varroa exposed (high DWV) pupae (solid line) or control (Varroa naïve, low DWV) pupae (dotted line). Error bars show the standard error of the mean.

The control lines in Figure 5.4 show the natural fluctuations in expression for the selected Hox gene during the delicate process of honey bee pupation and metamorphosis. There are subtle but significant differences at certain sampling ages between the control group and the *Varroa* exposed, virus infected individuals;

- For distalless (Dll) (Figure 5.4 A) there was no significant difference in the expression levels in control bees and DWV infected bees, with the exception of pupae sampled at 5 days post capping. In these individuals, the expression of Dll was detected as being significantly higher in the control group ($t(15)=2.442$, $p=0.027$).
- Expression levels of extradenticle (Ext) (Figure 5.4 B) showed significant differences from the control group in DWV infected bees at 6 days post capping ($t(13)=2.432$, $p=0.03$).
- In the sampled bees, expression levels of buttonless (Btn) (Figure 5.4 C) were not significantly different between the treatment groups, with the exception of individuals sampled at 7 days post capping, where *Varroa* exposed pupae had significantly lower levels of Btn ($t(16)=2.911$, $p=0.01$).
- AbdominalA (AbdA) (Figure 5.4 D) expression levels again followed a similar pattern in control and DWV/*Varroa* exposed pupae, but in individuals sampled at days 5 ($t(16)=2.482$, $p=0.025$) and 6 ($t(11)=3.245$, $p=0.008$) post capping, there was a significant difference in the measured levels of AbdA in the *Varroa* exposed pupae in comparison with the control group.
- ExtraExtra (ExEx) (Figure 5.4 E) expression levels remained consistent between the two groups of pupae, with no significant differences ($p>0.05$). *Varroa* exposure and DWV infection appeared to have no impact on the pattern on ExEx expression in these pupae.
- For drop1 (Dr1) (Figure 5.4 F) the expression levels were not significantly different ($p>0.05$) in control bees when compared to DWV infected bees, with the exception of pupae sampled at 6 days post capping. In these individuals, the

expression of Dr1 was detected as being significantly lower in the control group ($t(11)=3.635$, $p=0.004$).

The results of this sampling experiment indicate that *Varroa* parasitism and/or DWV infection can influence the intricate expression patterns of developmental genes during honey bee pupation. However, the disturbances measured in this set of sampled pupae were not as pronounced or as universal across large groups of Hox genes as those observed in the microarray experiment conducted previously. There are two reasonable hypotheses for this inconsistency: Either the dissimilarities in expression pattern alterations are caused by the experimental method, as sampling of naturally infested pupae does not allow control of the viral dose, of the exact timing of the onset of *Varroa* feeding, the strain of DWV present within the colony or the environmental conditions experienced by the pupae prior to sampling. Alternatively, it could be that the honey bee transcriptional response to *Varroa* feeding or DWV infection is influenced by the honey bee genotype, and the pupae sampled in the two separate experiments have differing responses due to their genetic dissimilarities.

A number of further experiments were therefore conducted, firstly using direct injection of DWV to allow for closer control of the viral dose, followed by a comparison of the transcriptional response of honey bee pupae from a range of colonies using this more controllable technique.

5.3.2 Analysis of developmental gene expression in experimentally DWV injected honey bee pupae

Injection of DWV VP into ‘white eye’ pupae can be used to simulate DWV exposure through *Varroa* feeding, as described in Chapter 3 of this work. An experiment was done in which individual ‘white eye’ pupae from a single colony were given a single infectious dose of DWV (1×10^{-5} ng viral RNA) by injection, and then samples taken at regular intervals to reveal more about how DWV infection affects developmental gene expression

in honey bee pupae. This experiment was done twice, using honey bees taken from a different colony each time.

Experiment 1.

This experiment was done on 19.7.2016

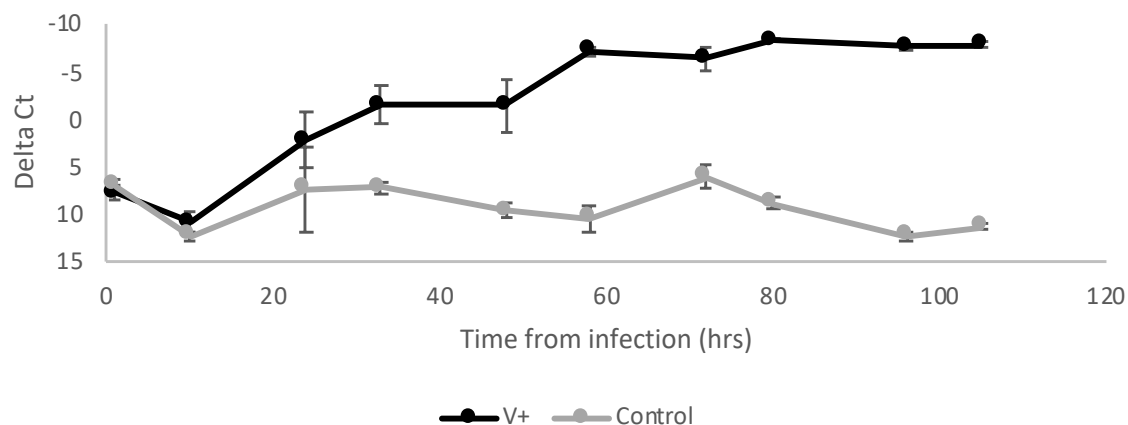


Figure 5.5. Experiment 1 - Comparative levels of DWV in honeybee pupae injected with DWV RNA (V+) or with a PBS control injection (Control) as shown by qPCR deltaCt. Linear regression fitted, shown as dotted lines. Samples collected at 1,10, 24, 48, 72, 80 96 and 105 hours post injection.

Once it had been established that the DWV injection had led to the expected DWV infection in V+ bees (Figure 5.5), qPCR was used to investigate the impact on this infection on the expression profiles of a number of developmental genes.

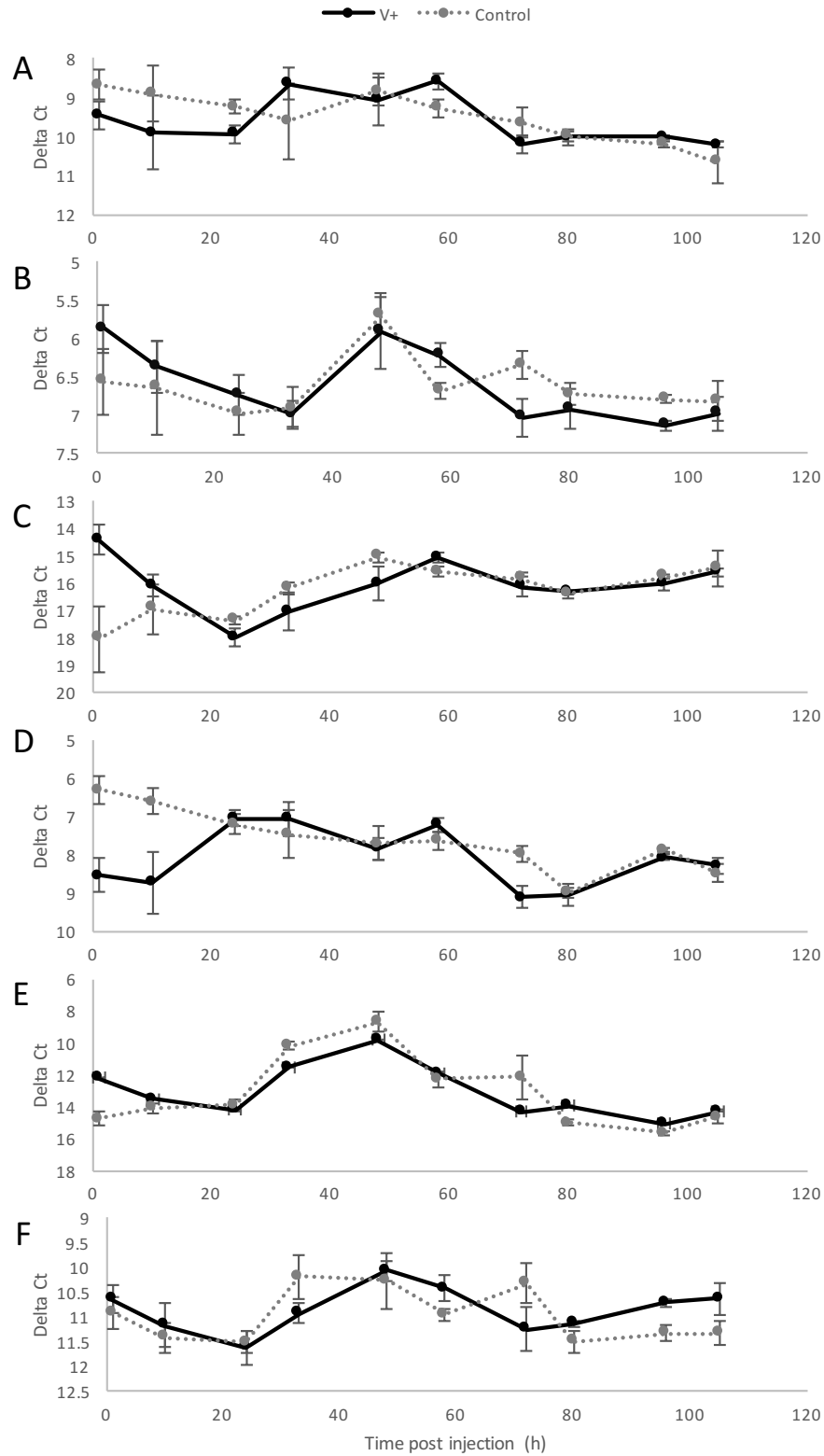


Figure 5.6 qPCR results, revealing the pattern of gene expression in 6 Hox genes in experimentally DWV injected pupae: A. *Distalless* (*Dll*). B. *Extradenticle* (*Ext*). C. *Buttonless* (*Btn*). D. *Abdominal A* (*AbdA*). E. *ExtraExtra* (*ExEx*). F. *Drop1* (*Dr1*). Mean expression levels, normalized to actin (Delta Ct) are shown for DWV injected pupae (solid line) or control (control injection) pupae (dotted line). Error bars show the standard error of the mean.

The control lines in Figure 5.6 show the fluctuations in expression for the selected Hox gene during the pupal to adult transition in pupae that have been removed from the colony and given a control injection. There are subtle but significant differences at certain sampling points between the control group and the DWV exposed, virus infected individuals;

- There were no significant differences between the treatment groups in the expression of Dll. The expression patterns were broadly similar in the virus injected and control injected pupae (Figure 5.6 A).
- Expression levels of ExEx showed no significant differences between the treatment groups, until the 58 hour post injection (h.p.i) sampling point, when levels are significantly higher in DWV infected pupae ($t(10)=2.241$, $p=0.049$), and at the 96 h.p.i sampling point where the control pupae have higher ExEx expression ($t(9)=3.72$, $p=0.007$) (Figure 5.6 B).
- Btn levels appear to be affected by the very early stages of DWV infection, with a significant difference between the levels in the two treatment groups at the first sampling point ($t(9)=2.753$, $p=0.025$) (Figure 5.6 C).
- Similarly, expression of AbdA are perturbed immediately after DWV injection at the 1 h.p.i sampling point ($t(8)=3.852$, $p=0.005$). However, unlike Btn, AbdA expression levels also appear to be disturbed by DWV infection at later time points, including the 72 h.p.i sampling point ($t(9)=2.821$, $p=0.02$) (Figure 5.6 D).
- ExEx expression is different according to treatment group at 1 h.p.i ($t(8)=4.457$, $p=0.002$), but the expression levels then showed no significant difference ($p>0.05$) between the treatment groups for all subsequent sampling points (Figure 5.6 E).
- In contrast, Dr1 expression levels are unaffected by DWV injection at the early sampling points, with no significant difference ($p>0.05$) between the treatment groups, but there appears to be a significant impact of DWV infection at the later sampling points – 96 h.p.i ($t(7)=3.385$, $p=0.012$).

Along with Hox genes, the ecdysone signalling pathway is known to play a crucial role in post embryonic development and caste determination in honey bees. The transcription levels of the ecdysone receptors EcR-A and EcR-B have been shown to fluctuate during honey bee pupation and to be linked to the titre of 20-hydroxyecdysone (20E) (Mello, *et al.*, 2014). Here we have compared the expression levels of the two ecdysone receptors (EcR-A and B) and two members of the ecdysone signalling pathway crooked legs (Crol) and insulin like peptide receptor (InR) in honey bee pupae injected with either DWV RNA or a virus free control injection.

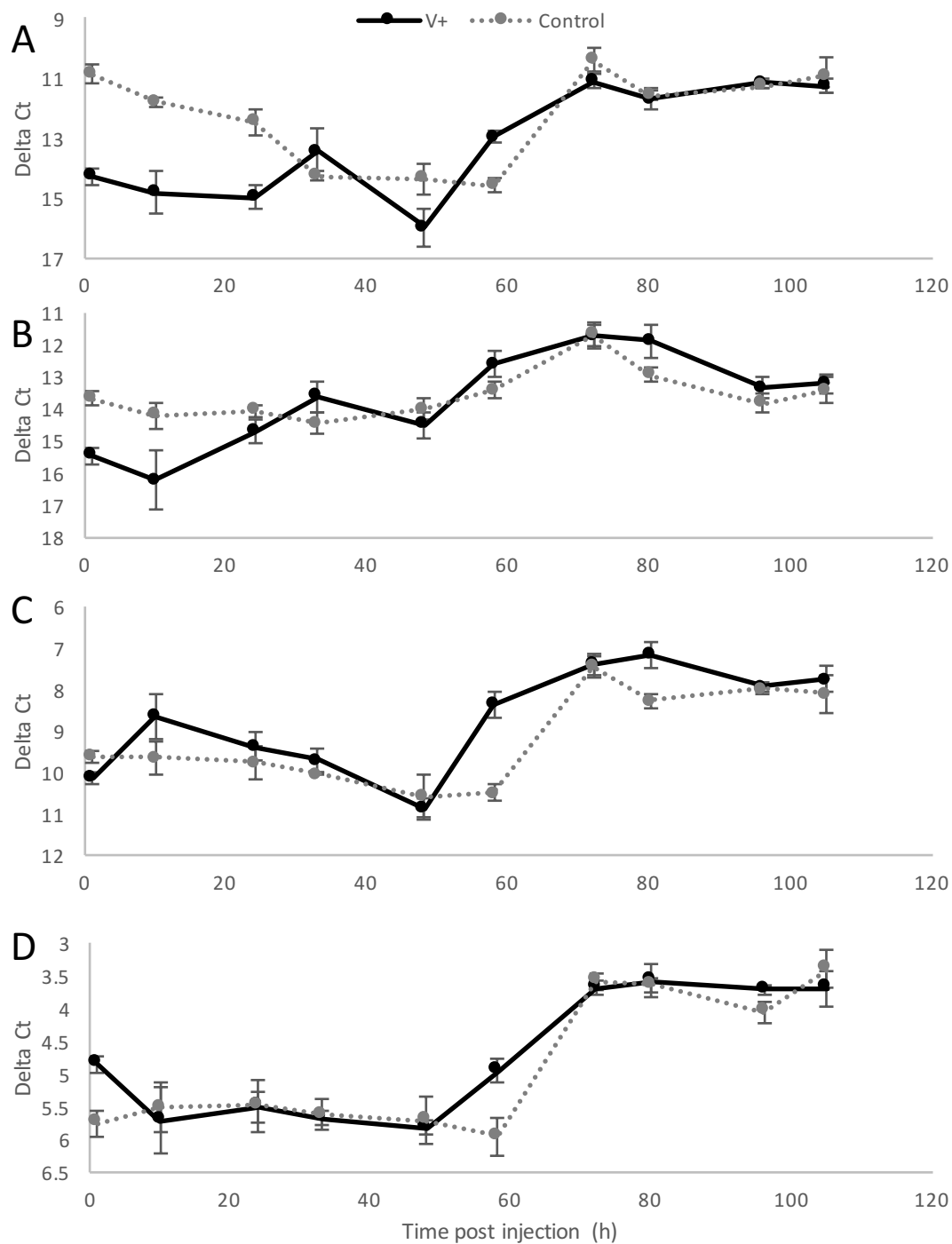


Figure 5.7. qPCR results, revealing the pattern of gene expression in 4 ecdysone signaling pathway genes in experimentally DWV injected pupae: A. *Apis mellifera* ecdysone receptor transcript variant A (EcR-A). B. *Apis mellifera* ecdysone receptor transcript variant B. C. Insulin like peptide receptor (InR). D. Crooked legs (Crol). Mean expression levels, normalized to actin (Delta Ct) are shown for DWV injected pupae (solid line) or control (control injection) pupae (dotted line). Error bars show the standard error of the mean.

As with the Hox genes, the tested genes from the ecdysone signalling pathway were found to have different expression patterns in DWV injected honey bee pupae, when compared to pupae given a control injection. The main impacts of DWV infection are outlined below:

- EcR-A expression is strongly impacted by early DWV infection, with a significantly lower expression level in injected pupae sampled at 1 h.p.i ($t(7)=3.945$, $p=0.006$), 10 h.p.i ($t(6)=4.138$, $p=0.006$) and 24 h.p.i ($t(7)=3.997$, $p=0.005$). The expression pattern then appears to be similar in the infected and control groups, with the exception of a significant perturbation at 58 h.p.i ($t(10)=5.080$, $p<0.001$) (Figure 5.7 A).
- EcR-B expression levels were observed to be significantly lower in DWV infected bees at 1 h.p.i ($t(8)=5.777$, $p<0.001$), with the expression pattern appearing to be largely unaffected by DWV at later sampling points (Figure 5.7 B).
- InR follows a similar expression pattern in both DWV infected and control pupae until the 58 h.p.i sampling point, when the levels in infected bees are significantly higher than those found in the control individuals ($t(9)=4.626$, $p=0.001$). There is also a difference in the expression of InR detected at the 80 h.p.i sampling point ($t(9)=2.427$, $p=0.038$)(Figure 5.7 C)
- Crol expression appears to be affected by the early stages of DWV infection (1 h.p.i ($t(8)=3.919$, $p=0.004$) but then the infected and control pupae show similar levels of expression until the 58 h.p.i sampling point, where they diverge ($t(10)=3.197$, $p=0.010$).

This experiment has allowed greater control of viral dose, pupal conditions and sampling times and it has therefore allowed us a greater insight into the effect that DWV infection has on developmental gene expression. However, all of the individuals used in the experiment were worker pupae from a single colony at a single time point and can therefore be assumed to be closely genetically related (Blanchetot, 1991). In order to determine how reproducible, the results are across genetically distant honey bees the experiment was repeated with a different source colony as follows.

Experiment 2.

The honey bee pupae used for the repeat injection and sequential sampling experiment were susceptible to DWV infection, with the levels of DWV increasing rapidly in DWV injected pupae during the first 60 hours of the experiment (Figure 5.8). The progression of the DWV infection appears to follow a similar pattern, in terms of virus levels per pupae at each sampling time point.

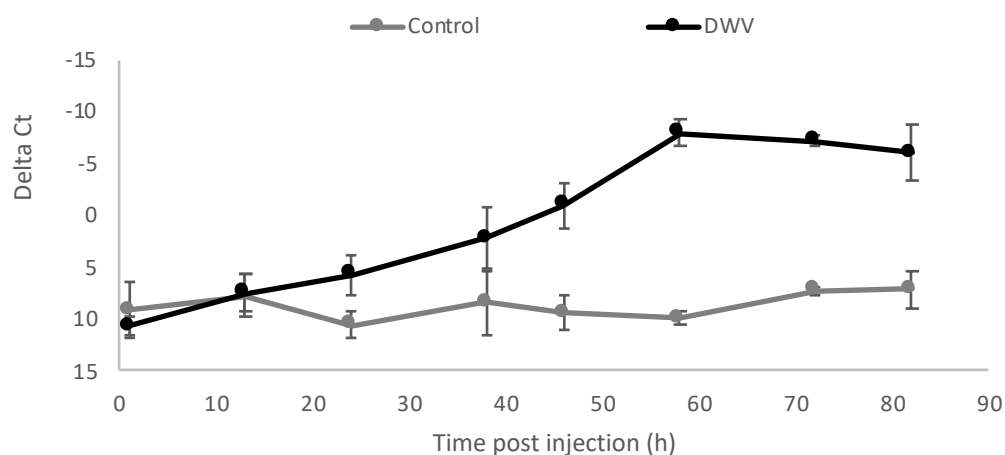


Figure 5.8 Experiment 2 - Comparative levels of DWV in honeybee pupae injected with DWV RNA (V+) or with a PBS control injection (Control) as shown by qPCR deltaCt. Linear regression fitted, shown as dotted lines. Samples collected at 1, 10, 24, 48, 72 and 80 hours post injection.

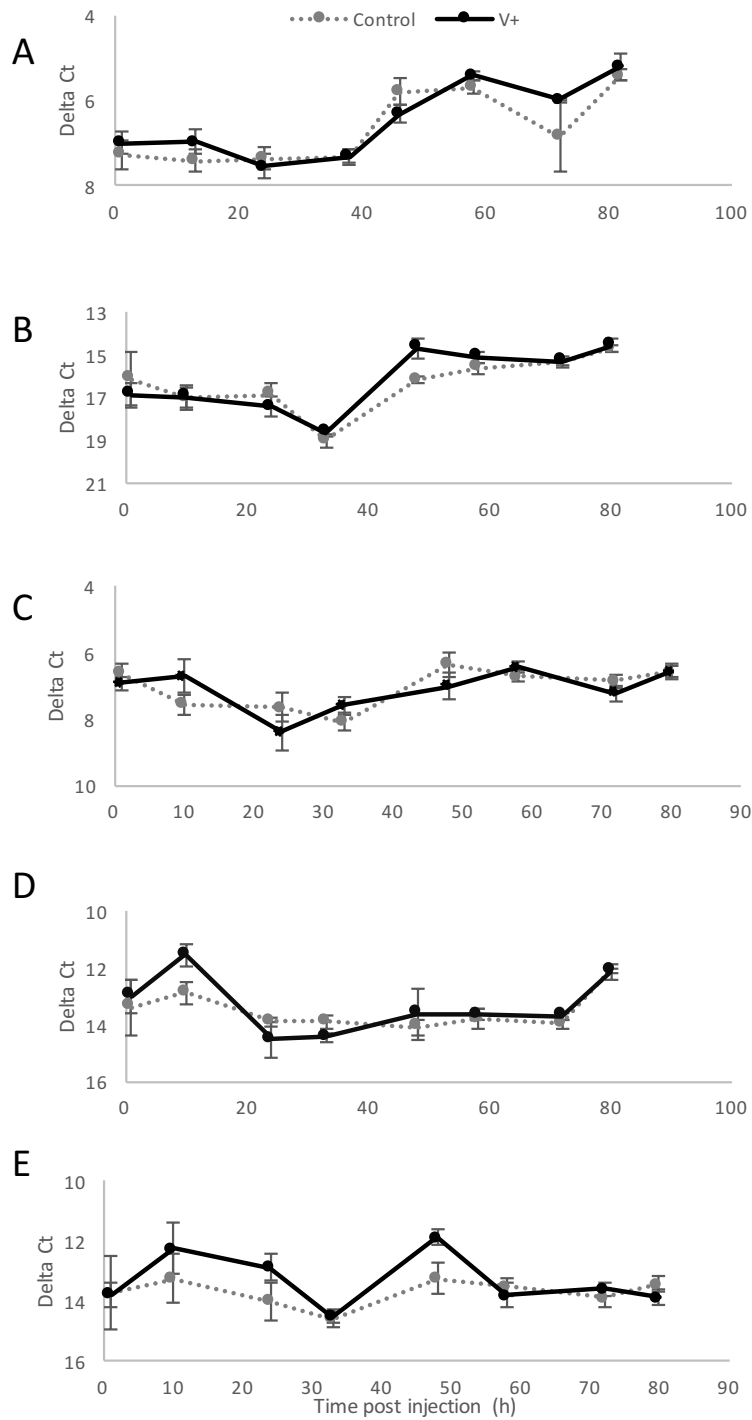


Figure 5.9. qPCR results, revealing the pattern of gene expression in Hox and ecdysone signalling pathway genes in experimentally DWV injected pupae.; A. Extradenticle, B. Buttonless, C. AbdominalA, D. *Apis mellifera* ecdysone receptor transcript variant A (EcR-A), E. *Apis mellifera* ecdysone receptor transcript variant B. Mean expression levels, normalized to actin (Delta Ct) are shown for DWV injected pupae (solid line) or control (sham injection) pupae (dotted line). Error bars show the standard error of the mean.

The impacts of DWV infection on pupal developmental gene expression in the second experiment are outlined below:

- Expression levels of ExEx remain similar in both treatment groups, with no significant impacts of DWV infection detected in this sample set ($p>0.05$) (Figure 5.9 A).
- In this sample set, Btn levels appear to be unaffected by DWV infection until the 48 h.p.i sampling point, where the expression level appears to be significantly increased in the DWV infected individuals ($t(9)=2.76$, $p=0.022$). At later sampling points the detected levels of Btn are similar in both treatment groups (Figure 5.9 B).
- AbdA expression does not appear to be affected by DWV infection in these pupae (Figure 5.9 C), with no significant difference between the expression in the two treatment groups ($p>0.05$).
- Expression of Ecr-A is disturbed by early stage DWV infection in these pupae, however, unlike the pupae in the first experiment, in these bees the disturbance is at 10 h.p.i and here expression in the DWV infected pupae is significantly higher than the control group ($t(10)=2.413$, $p=0.037$).
- EcR-B expression is unaffected by the early stages of DWV infection, but is significantly higher at 48 h.p.i in DWV infected individuals ($t(9)=2.626$, $p=0.027$).

As described in the first injection experiment, the DWV infection does appear to disturb the pattern of expression of certain developmental genes, the timeframes and magnitude of the disturbances are not consistent between the two experiments. This indicates that viral replication may be leading to gene expression deregulation rather than a controlled, directional alteration in the expression of these crucial developmental genes. The genetic differences between the two sampled colonies may be the basis of the differing responses observed in the two sets of samples.

When compared to the previous injection experiment, the magnitude and direction of the disturbances to developmental gene expression appear to be specific to each sampled

colony of bees rather than a universal, directional response to DWV infection. There are also some differences between the ‘control’ expression patterns for the genes, but in general they are far more similar between the experiments than those observed in the infected pupae.

5.3.3 Multi colony injection experiment

The results of the previous experiment (5.3.2) indicate that, whilst developmental gene expression in honey bee worker pupae is perturbed by DWV infection, the actual magnitude and direction of the disturbance to each tested gene appears to be fairly consistent within a colony of genetically related individuals but to differ between colonies. In order to further explore this observation, a larger scale injection experiment was carried out (5.2.3).

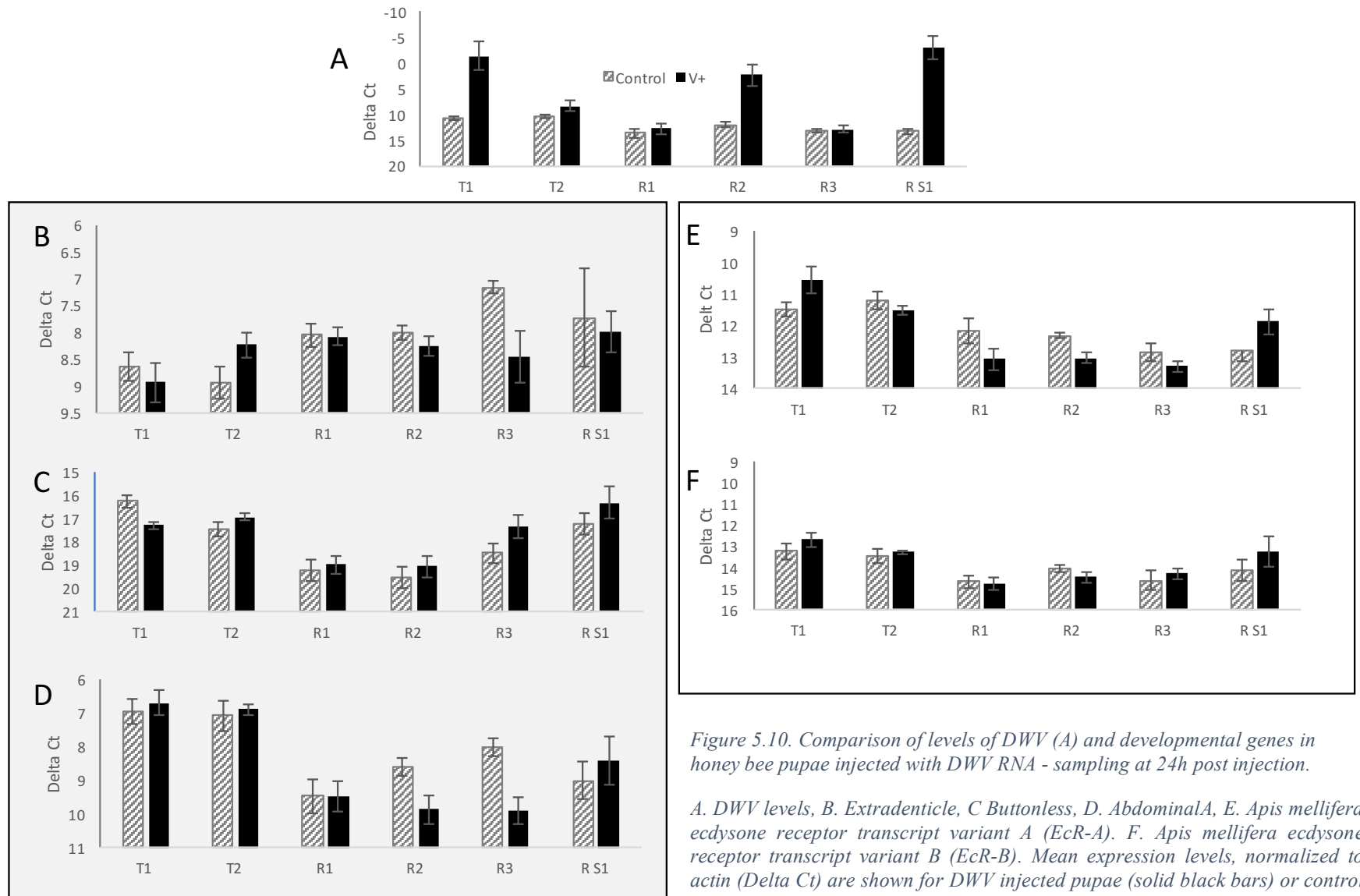


Figure 5.10. Comparison of levels of DWV (A) and developmental genes in honey bee pupae injected with DWV RNA - sampling at 24h post injection.

A. DWV levels, B. Extradenticle, C. Buttonless, D. AbdominalA, E. *Apis mellifera* ecdysone receptor transcript variant A (EcR-A), F. *Apis mellifera* ecdysone receptor transcript variant B (EcR-B). Mean expression levels, normalized to actin (Delta Ct) are shown for DWV injected pupae (solid black bars) or control (control injection) pupae (grey hashed bars). Error bars show the standard error of the mean.

5.3.3.1 Sampling at 24h Post Injection

Honey bee pupae from 6 colonies, spread over 2 apiaries, were sampled and age matched, *Varroa* free pupae were injected with a single infectious dose of DWV or with a virus free control injection. Half of the injected pupae were sampled after 24h and, following RNA extraction and processing, qPCR was used to compare the levels of DWV and selected developmental genes in the infected and control pupae.

In Figure 5.10 A the DWV levels in pupae at the 24h sampling point are shown. It appeared that some colonies (T1, R2, and RS1) are more susceptible to DWV, with the infection in the DWV injected pupae yielding increased levels of DWV than those found in the control pupae at the 24h sampling point. The other colonies (T2, R1 and R3) did not appear to show elevated DWV levels following DWV injection at this 24h post injection sampling, however at the later (96h) time point (Fig 5.11 A) the DWV levels are significantly elevated in all colonies. A two tailed ANOVA indicated that, 24 hours post DWV infection, there was a significant difference in viral outcome between the DWV injected and control pupae ($F(1,59)=83.365$, $p<0.001$). There was also a statistically significant effect of parent colony on DWV levels ($F(5,59)=16.366$, $p<0.001$), and a significant interaction between the effects of treatment (DWV injection or control injection) and source colony on the DWV levels in the sampled pupae $F(5,59) = 13.603$, $p<0.001$). This result reveals that the progression of DWV infection differs between colonies, even when the dose, time of injection, and strain of DWV are experimentally controlled.

As we hypothesized following the previous results from two colonies, the expression of the selected developmental genes is altered by DWV infection, with clear variability between colonies. The main results from the 24 h sampling point are outlined below:

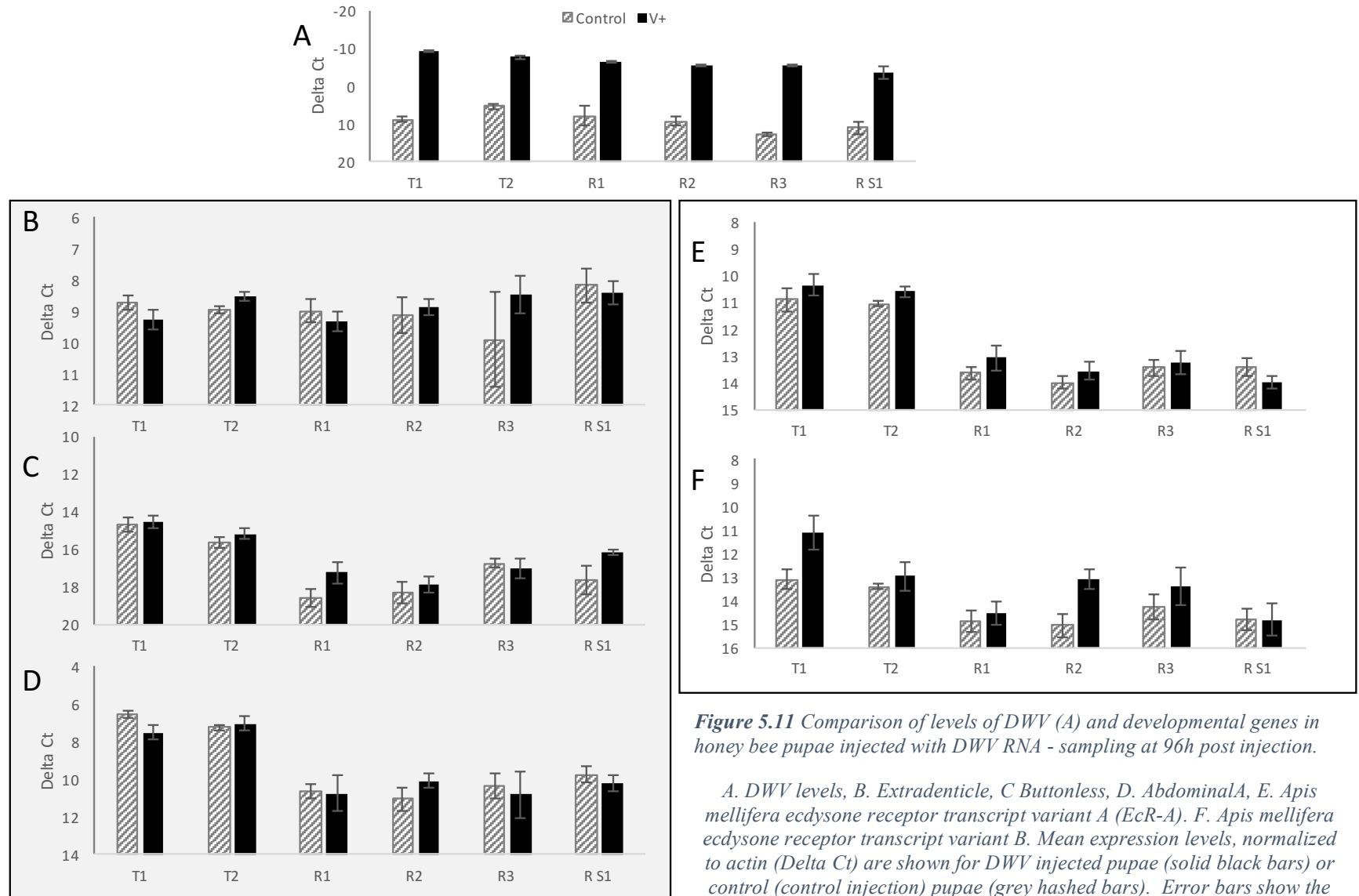
- Exd expression levels do not show a significant interaction between the effects of treatment and source colony ($F(5,60) = 1.414$, $p=0.232$). There was also no significant effect of DWV injection compared to the sham injection (F

(1,60)=1.219, $p=0.274$) and no significant differences between the responses of the source colonies ($F(5,60)=2.243$, $p=0.061$). Post hoc testing showed that only samples from colony R3 showed a significant difference in Exd expression levels between treatment groups at this sampling point ($t(10)=2.563$, $p=0.028$).

- The expression of Btn does differ significantly between the sample colonies at the 24h sampling point ($F(5,60)=14.452$, $p<0.001$), but there was no difference between the two treatment groups ($F(1,60)=2.343$, $p=0.131$). Btn expression levels do not show a significant interaction between the effects of treatment and source colony ($F(5,60) = 1.660$, $p=0.158$). However, post hoc testing shows that one colony - T1 ($t(10)=3.343$, $p=0.007$ – does have a significant alteration in Btn expression in DWV injected pupae, when compared to sham injected.
- AbdA expression differs significantly between the sample colonies at the 24h sampling point ($F(5,60)=13.82$, $p<0.001$). However there was no a significant difference in expression caused by the treatment ($F(1,60)=1.841$, $p=0.18$). The expression of AbdA does appear to show a significant interaction between effects of treatment and source colony ($F(5,60) = 2.485$, $p=0.041$). Post hoc testing shows that in this case two colonies, R2 ($t(10)=2.443$, $p=0.035$) and R3 ($t(10) = 3.836$, $p=0.003$) show significant alterations in AbdA expression following DWV inoculation, when compared to the expression in sham injected pupae from the same source colony.
- EcrA expression differs significantly between the sample colonies at the 24h sampling point ($F(5,60)=15.216$, $p<0.001$). However there was no a significant difference in expression caused by the treatment ($F(1,60)=0.237$, $p=0.628$). EcrA expression also shows a significant interaction between effects of treatment and source colony ($F(5,60) = 3.796$, $p=0.005$), however, when considered alone, the differences in expression in the individual colonies are not consistent enough to be considered significant.
- EcrB expression differs significantly between the sample colonies at the 24h sampling point ($F(5,60)=6.541$, $p<0.001$). However there was no a significant difference in expression caused by the treatment ($F(1,60)=1.186$, $p=0.281$). EcrB expression does not show a significant interaction between the effects of treatment

and of source colony ($F(5,60) = 0.718$, $p=0.613$) and the expression levels measured in both the treatment groups are not significantly different in any of the tested colonies.

There is no clear distinction between the transcriptional responses shown by the rapidly susceptible to DWV colonies (T1, R2, and RS1) and the other colonies which had low DWV at this sampling point.



5.3.3.2 Sampling at 96h Post Injection

Figure 5.11 A shows the levels of DWV and the selected Hox and EcR genes in pupae samples 96h after injection. In contrast to the earlier (24 h) sampling point, there is a significant DWV infection in pupae from all colonies by 96 h post injection with DWV. A two tailed ANOVA reveals that, 96 hours post DWV infection, there is a significant effect of both treatment ($F(1,59)=306.084$, $p<0.001$) and source colony $F(5,59)=92.255$, $p<0.001$). There is also a statistically significant interaction between the effects of treatment (DWV injection or control injection) and source colony on the DWV levels in the sampled pupae $F(5,59) = 29.528$, $p<0.001$. From this we can conclude that, although the infections progress at different rates according to source colony, all of the colonies sampled were ultimately susceptible to experimental DWV infection.

There was some disturbance to the expression of the developmental genes at this sampling point, although less than that observed at the 24h point. Again, we do observe variability in the responses of pupae from different source colonies.

- The expression of Exd was significantly influenced by the source colony of the pupae ($F(5,58)=8.756$, $p<0.001$) but not by the experimental treatment ($F(1,59)<0.001$, $p=0.995$). Exd expression levels do not show a significant interaction between the effects of treatment and source colony ($F(5,58) = 0.646$, $p=0.666$). Post hoc testing did not indicate any significant differences between the control and treatment groups in any of the sampled colonies.
- The expression of Btn was significantly influenced by the source colony of the pupae ($F(5,58)=26.741$, $p<0.001$) but not by the experimental treatment ($F(1,58)<1.241$, $p=0.270$). Btn expression levels do not show a significant interaction between the effects of treatment and source colony ($F(5,58) = 0.522$, $p=0.759$). Again, post hoc testing did not indicate any significant differences between the control and treatment groups in any of the sampled colonies.
- The expression of AbdA is significantly different between the sample colonies ($F(5,58) = 26.038$, $p<0.001$) but not between treatment groups ($F(1,58)=0.001$,

$p=0.974$). AbdA expression does not appear to show a significant interaction between effects of treatment and source colony ($F(5,58) = 1.028$, $p=0.410$). Again, post hoc testing did not indicate any significant differences between the control and treatment groups in any of the sampled colonies.

- EcrA expression also fails to show a significant difference in expression due to treatment group at 96 h ($F(1,58) = 2.571$, $p=0.114$) but there is a significant effect of source colony ($F(5,58) = 35.244$, $p<0.001$). No interaction was found between effects of treatment and source colony ($F(5,58) = 0.618$, $p=0.687$), and any differences between the control and treatment groups were not significant.
- EcrB expression is significantly affected by both source colony ($F(5,58) = 9.211$, $p<0.001$) and by the treatment group ($F(1,58) = 8.312$, $p=0.006$). In contrast to the other genes analysed at this time point, EcrB expression does show a significant interaction between effects of treatment and source colony ($F(5,58) = 2.550$, $p=0.037$), with expression of EcrB being significantly increased in DWV infected individuals from colonies T1 ($t(9)=2.410$, $p=0.039$) and R2 ($t(10)=3.088$, $p=0.011$).

5.4 Discussion

The results of this chapter suggest that, whilst DWV infection may alter the expression patterns of key developmental genes in infected honey bee pupae as they undergo morphological development to the adult life stage, the outcome of the effect of DWV infection is complex and depends on a range of variables including bee colony and pupal age. The experiments described in this chapter may begin to illuminate the interaction between DWV infection and honey bee developmental genes, but the outcomes of the experiments were too variable to allow us to draw firm conclusions.

To our knowledge, this chapter represents the first published report of the effect of DWV infection on the expression of honeybee developmental genes. This is surprising, given that the main effect of DWV infection is to cause developmental abnormalities in *Varroa*-parasitized bees. However, it may also reflect the technical challenges involved in this area of study.

Little is known about the precise molecular mechanisms that underpin the normal developmental transition from honeybee larvae to the adult form, and the main focus of published work on gene expression during honey bee development has been queen-worker dimorphism (Evans and Wheeler, 1999; Evans and Wheeler, 2001) and on perturbations to gene expression following *Varroa* parasitism (Navajas, *et al.*, 2008; Kuster, *et al.*, 2014). As outlined in the introduction to this chapter, what we do know about the control of larval-to-adult insect metamorphosis indicates that the ecdysone signalling pathway, controlled by changes in the titre of the hormone 20-hydroxyecdysone (20-HE), detected by an ecdysone receptor protein complex (ECR-C) plays a crucial role in the control of honey bee metamorphosis and pupal development (Barchuk, *et al.*, 2008; Gauhar, *et al.*, 2009). Insect morphological development is also known to be regulated by a suite of evolutionary conserved homeodomain proteins, regulating cell fate and cell-type gene expression to determine insect segment morphology and the development of appendages, organs, the nervous system and the neuroendocrine systems (Hayashi and Scott, 1990; Ladam and Sagerstrom, 2014).

The research in this chapter was based on preliminary results from a honey bee microarray experiment done by our research group, in which gene expression was quantified for individual pupae of different ages sampled from a single bee colony and naturally parasitized with different numbers of *Varroa* mites (data not shown). This data led us to hypothesise that DWV infection, alongside *Varroa* parasitism, resulted in a significant, directional perturbation to developmental gene expression. However, the results from this chapter point to a more complex situation, in which experimental DWV infection has been shown to cause subtle up or down regulation of developmental gene expression, but these disturbances are transient, occurring only during certain sampling points, and appear to differ depending on the source colony of the experimental bees. Both the direction and magnitude of the disturbance to gene expression appear to be dependent on the sampled colony. This is best illustrated by looking at the expression of a single gene, *EcrA*, across the different injection experiments:

EcrA is one of two ecdysone receptors found in honey bees, transcription of which is closely linked to honey bee developmental stage and caste, and to be inversely linked to the titres of the hormone 20-hydroxyecdysone (20-HE) (Mello, *et al.*, 2014). *EcrA* has been shown to be the predominantly expressed ecdysone receptor during postembryonic development, and to exhibit major differences in expression according to honey bee caste during the larval/pupal metamorphic molt (Mello, *et al.*, 2014).

- In the initial, single colony injection time course experiment (Section 5.3.2, Figure 5.6 A) the expression of *EcrA* was significantly reduced in individuals injected with DWV when compared to the control pupa during the first 24 h post injection, but at the later sampling points (36 h.p.i onwards) the levels of *EcrA* detected in the control and infected pupae followed a similar expression pattern. This experiment was then done again, using pupae from a different source colony and injected with the same DWV virus preparation. In this second experiment, a

disturbance to the expression pattern of EcrA was observed shortly after DWV injection, but the disruption was restricted to the 10 h.p.i sampling point.

- At the first (=24 h) sampling point of the multi colony injection experiment (Section 5.3.3.1) the expression of EcrA within pupae was affected by the presence of infecting DWV, with EcrA expression higher than the control pupae in some colonies (RS1) and lower in others (R2). The results also indicate that a larger sample size would have allowed us to identify a significant up or down regulation in a number of the other colonies. At the 96 h.p.i time point no colonies showed significant differences in EcrA expression between the control and experimental groups, this result is in line with the earlier experiments where the transcriptional response in infected bees was restricted to the earlier sampling points.

These results effectively illustrate the between-colony variation in transcriptional response to DWV infection in honey bees. All of the experiments described in the previous paragraph used the same strain of DWV for inoculation and the same experimental design in terms of age of bee pupa, dose of DWV, treatment of samples and processing of sampled insects. Therefore, it is reasonable to suggest that the variation in response must be caused by between-colony variation in the bees sampled. This variation could be due to the genetic differences between colonies of bees or it could be due to differences in environment (such as the nutritional status or forage abundance experienced by each colony, the climatic conditions or temperature experienced by the experimental individuals after laying but prior to their collection for injection or the predominant strain of DWV or other disease within each source colony). The fact that the multi colony injection experiment involved sampling from bees within a small area on the same day (and therefore with very similar climatic and forage conditions) removed some of this variability, but we still cannot be sure of the real cause of this result without further experiments. However, the results from this study have opened up the way forward for understanding the effects of DWV infection on honeybee development.

One testable hypothesis is that different honeybee genotypes show different responses in developmental gene expression to DWV infection. Genotypic variation in transcriptional responses to parasite infection has been observed in other insects. For example, it has been shown that different strains of the mosquito *Aedes Aegypti* have very distinctive transcriptional responses to infection with dengue virus, with only 5% of the differentially expressed genes in experimentally infected mosquitos being common to both strains of the insect used in the study (Behura, *et al.*, 2014). Meanwhile, bumblebee (*Bombus terrestris*) genotype has also been shown to determine the host response to infection with the trypanosome gut parasite *Crithidia bombi*, leading to some bumblebee genotypes being more susceptible to the parasite (Barribeau, *et al.*, 2014).

There have been a number of studies quantifying the honey bee transcriptional response to viral infection, notably (Ryabov, *et al.*, 2014; Ryabov, *et al.*, 2016; Brutscher, *et al.*, 2017; Doublet, *et al.*, 2017) but none of these have gone on to compare these responses in bees with different genotypes. For example, the Ryabov *et al.* (2014, 2016) papers focused on individual honey bees from a single colony, so no between-colony variation was taken into account. The scope of the Ryabov *et al.* work did not allow genotyping of these individual honey bee samples, which would have been necessary to determine if the divergent transcriptional responses and susceptibility to DWV infection were due to differences in the genotypes of the bees in the study. A meta-analysis study by Doublet *et al.* (2016) of many published and newly conducted honey bee transcriptome projects, allowed the identification of common molecular responses to major parasites and pathogens; this approach is useful for revealing responses conserved across geographically, temporally (and therefore presumably genetically) diverse bees, but as a consequence of this, discrepancies due to genetic variation are ignored or obscured. The results described in this chapter highlight how subtle or inconsistent variability in transcriptional responses can be missed by widely used microarray techniques and by experimental designs involving pooled or closely genetically related individuals.

The results of these experiments allow us to conclude that DWV infection can subtly alter the expression patterns of key developmental genes in infected honey bee pupae, but could these subtle, variable alterations be responsible for the consistent, well characterised deformities that are known to be the result of DWV infection, and that have been shown to occur in pupae injected with DWV using the experimental system employed in this chapter (Chapter 3, Section 3.3.3.2)? It has been experimentally demonstrated that disruption to Hox gene expression can result in dramatic defects in the development of the nervous system or physical structure of the experimental animal. In *Drosophila*, knocking out Hox genes causes homeotic transformations, for example the antennapedia mutants, where loss of function of the gene results in one leg pair being replaced by ectopic antennae. Overexpression of antennapedia (gain of function) transforms the antenna into ectopic legs (Pearson, *et al.*, 2005). However, there is very little known about the effects of virus infection on Hox gene expression, and the consequences for the development of the infected individual. The other developmental pathway indicated to be affected by DWV infection, the ecdysteroid signalling pathway, has previously been shown to be susceptible to manipulation by virus infection in insects. As described in the introduction to this chapter, infection with the baculovirus *Lymantria dispar* nuclear polyhedrosis virus affects the development of its insect hosts *Lymantria dispar* (the gypsy moth) and *Spodoptera frugiperda* (the fall armyworm) by altering the levels of ecdysteroid, the insect moulting hormone, in the haemolymph of infected individuals (Burand and Park, 1992; Park, *et al.*, 1993; Park, *et al.*, 1996)

Further experiments are required to establish if the novel results outlined in this chapter could provide an explanation for the characteristic deformities observed in DWV infected honey bees. Experimental manipulation of the expression of Hox and ecdysone responsive genes in developing pupae would give a clearer indication of the impact that disturbances to these pathways has on honey bee development. This could be achieved through RNAi-mediated gene knockdown. This technique has been used to effectively suppress developmental and regulatory gene expression in adult honey bees, where injection of targeted dsRNA reduced the expression of both vitellogenin (vg) which encodes a precursor of the egg yolk proteins and is crucial in the caste differentiation of

honey bee larvae, and ultraspiracle (*usp*) which encodes a juvenile hormone (JH) receptor and is thought to be a transcription factor, mediating responses to JH (Corona, *et al.*, 2007; Ament, *et al.*, 2012; Wang, *et al.*, 2013).

The experiments described in the chapter were restricted by the need for age matched, *Varroa* free pupae, from specific honey bee colonies. There are always a limited number of suitable individuals within a colony at any one time. The result of this restriction was that it was not possible to collect a pre injection sample (0 h p i) or a no injection control for our experiments. Our results indicate that many of the perturbations to developmental gene expression occur within the first 48 h of DWV infection. It would be useful to conduct further experiments over a shorter time frame, freeing up pupae to add in these controls.

Chapter 6. Conclusions and future questions

For the past two decades, reports on declines in honey bee health and increases in colony losses have concerned beekeepers, scientists and the wider public (Meixner and Le Conte, 2016). There is a general consensus that both macro- and micro-parasites play a major role amongst the multifactorial catalogue of challenges faced by modern beekeepers (Potts, *et al.*, 2010; Ratnieks and Carreck, 2010; Vanbergen, *et al.*, 2013) and of particular concern is the invasive parasitic mite *Varroa destructor* and the microbial parasites that it vectors. *Varroa* has spread to most beekeeping areas of the world in the last 50 years and is one of the primary threats to honey bee health (Potts, *et al.*, 2016).

One of the *Varroa*-vectored pathogens which has emerged as a significant threat to honey bee health is deformed wing virus (DWV), an RNA virus that has been shown to be closely associated with colony losses (de Miranda and Genersch, 2010; Moeckel, *et al.*, 2011). Deformed wing virus (DWV) is widespread in honey bees and usually present as a low level, asymptomatic infection. However, the transmission of DWV by *Varroa* to developing pupae causes markedly elevated levels of a near clonal virus population, leading to characteristic developmental abnormalities (de Miranda and Genersch, 2010; Ryabov, *et al.*, 2014). DWV has been a focal point for research since this association was uncovered, but significant gaps remain in our understanding of pathology of *Varroa*-mediated DWV infections. This thesis attempts to address some of those knowledge gaps.

The aim of this project was to address three main research questions, revealing more about the interactions between honey bees, *Varroa* and DWV. Firstly, I examined how the transmission route of DWV affects the virulence and tissue localisation of the virus, testing the hypothesis that DWV is more infectious when transmitted by *Varroa* because it is injected directly into the haemolymph, bypassing the host's immune defences and

gaining unobstructed access to a wider range of tissues, as shown by RNA-FISH imaging of the timecourse of DWV infection. Next, I focussed on a question that currently divides the scientific community, investigating how *Varroa* transmission of DWV affects the diversity and predominant strain of the virus within the honey bee hosts. The published literature on the subject shows that *Varroa* parasitism leads to a narrowing in DWV diversity that accompanies the documented increase in viral load and the appearance of symptomatic honey bees (Martin, *et al.*, 2012; Ryabov, *et al.*, 2014). This points towards a single strain of the virus having a selective advantage when transmitted by *Varroa*, but there is currently no agreement over the sequence identity of that strain. Two repeated sampling experiments were conducted to try and resolve these inconsistencies, looking at the change in DWV population at the forefront of the *Varroa* invasion in the UK, and attempting to track any changes in the predominant strain of the virus over a single season in a heavily *Varroa* infested honey bee colony. Finally, I have investigated the hypothesis that DWV infection during crucial stages of honey bee pupation and metamorphosis causes disturbances to the expression of developmental gene pathways, including ecdysone receptor signalling and homeobox developmental genes, leading to the characteristic DWV symptoms.

As hypothesised, DWV is significantly more virulent when administered via injection rather than orally, as revealed by the infectious dose of the virus required to produce a symptomatic outcome in our two experimental systems (injection or oral exposure). At a fundamental level, this finding explains why the spread of *Varroa* mites, the saliva of which contains DWV proteins (Zhang and Han, 2018) and is injected into the honey bee pupae to facilitate mite feeding (Kuster, *et al.*, 2014), has led to the documented increase in DWV associated pathologies in honey bees (Martin, *et al.*, 2013). The results outlined in Chapter 3 of this work also reveal more about the intricacies of early stage DWV infection in developing honey bee pupae. An infectious dose of DWV, when administered by injection, takes just 2 or 3 days to result in an infection that is detectable by qPCR or RNA-FISH, and this is the case whether the injection takes place at the white eye, purple eye or newly emerged adult stage of honey bee development. However, when an infectious dose is given orally, it takes longer for virus replication to

progress to a detectable level (6 or 7 days from oral exposure), while the infection itself does not progress until a specific stage in pupal development, the fifth moult transition from propupae to pupae (Stell, 2012), when the digestive system is broken down during metamorphosis. This suggests that – in the absence of *Varroa*, when DWV infection occurs orally - the larval gut forms an effective barrier against DWV infection that confines infectious virus particles to the midgut or in the epithelial tissues of the gut lining at pupation. The RNA-FISH imaging conducted also revealed some tissue specificity in early stage DWV infection, with the virus appearing to preferentially replicate in the developing compound eye and the layer of cells surrounding the brain tissue, the salivary glands and mouth parts and in the epithelial cells of the mid and hind gut. DWV replication within the brain tissue may be linked to the cognitive and behavioural abnormalities in DWV infected honey bees described by (Wells, *et al.*, 2016; Benaets, *et al.*, 2017). The rapid rate of increase of DWV titre within bees that have been inoculated by injection indicates, a priori, that the bee immune system is unable to control the replication of the virus when this route of infection takes place. This most likely reflects the co-evolutionary history of honey bees and DWV, as prior to the invasion of *Varroa*, the bee gut would have been the first immune barrier to DWV.

The results of the Ardnamurchan sampling experiment show a shift from a mixed population of viruses, predominantly VDV-like or VDV/DWV recombinants, towards a mainly DWV-like virus population. This result needs to be examined in the context of the literature on DWV strain virulence. Our previous research (Ryabov, *et al.*, 2014) led us to predict that, as the recombinant strain that dominates local apiaries was present in the *Varroa* free Ardnamurchan bees, this strain would have a selective advantage when transmitted by the colonising *Varroa* mites and would become the most prevalent strain following the arrival of *Varroa* into the area. However, this was not the case in the Ardnamurchan samples where, in apiaries where the *Varroa* became established, there was a significant shift towards DWV-like virus strains. This result is more in line with the shift in the DWV-like virus population in Hawaii following the introduction of *Varroa* (Martin, *et al.*, 2012; Brettell, *et al.*, 2017). Another study that needs to be

considered when interpreting our results is (McMahon, *et al.*, 2016a), where it was established that VDV-like viruses caused significantly faster mortality in injected honey bees than DWV-like viruses, and that VDV-like and VDV/DWV recombinant strains of the virus dominate in apiaries in the south of the UK, where *Varroa* mites have been established for longer. In light of these results, we can conclude that *Varroa* mediated transmission does exert a selection pressure upon the diverse DWV viruses within honey bee colonies, and that DWV-like strains appear to have a selective advantage in the early stages of *Varroa* infestation. However, it appears that sampling within the first few years of *Varroa* becoming established may not tell the full story, and the virus population dynamics are influenced by more factors than are taken into account by this early shift towards DWV-like strains. Tracking the DWV-like viruses present in Ardnamurchan in a longer-term study would allow us to see if the early increase in DWV-like viruses has created a stable population, or if the levels of VDV-like and recombinant strains increase after more years of *Varroa* parasitism, as appears to have occurred in southern areas of the UK.

The results of our Warwickshire single apiary sampling experiment show that, over the course of a single season, the composition of the DWV-like virus population within a single heavily *Varroa* infected colony, with a well-established mite population, appears to be stable, irrespective of *Varroa* levels or virus load. If we also take into consideration the results of a previous experiment conducted in the same apiary (Ryabov, *et al.*, 2014) where the most prevalent strain of DWV was revealed by qPCR and Illumina sequencing, it would appear that the predominant strain in *Varroa* exposed bees, a VDV/DWV recombinant, has remained stable across a number of years. One possible explanation is that this strain has a selective advantage in this system, preventing random drift or diversification of DWV-like viruses back towards the mixed population found in *Varroa* naïve bees. The next step towards determining why certain strains of the virus dominate following *Varroa* mediated transmission, would be to sequence the DWV-like virus populations within apiaries in different countries, including areas where *Varroa* is present and absent, with the aim of identifying a section of the viral genome that confers this selective advantage. This approach has been

instrumental in uncovering the molecular drivers behind emerging human viral diseases, such as the Zika virus (Zhu, *et al.*, 2016).

The techniques used to quantify and visualise the dynamics of early stage DWV infections outlined in Chapter 3 of this work could also be used to further investigate this question of virulent DWV strains, particularly if used in combination with a novel reverse genetic system (Lamp, *et al.*, 2016). Full length infectious clones of the published genomes of DWV, VDV and the VDV/DWV recombinant strain could be used for dose response and time course injection experiments to reveal more about how infectious each strain is when injected to mimic *Varroa* feeding. Competition experiments involving the injection of exact mixtures of the infectious clones, followed by strain specific qPCR, would show how the strains compete whilst replicating within honey bee tissue. If fluorescently tagged infectious clones were produced, as (Kummerer, *et al.*, 2012) successfully accomplished with the Chikungunya virus (a positive sense RNA virus, vectored by mosquitos), then the early stage infection dynamics of the different viral genomes could be visualised and compared.

The work described in Chapter 5 supports our hypothesis that DWV infection during crucial stages of honey bee pupation and metamorphosis causes disturbances to the expression of developmental gene pathways, including ecdysone receptor signalling and homeobox developmental genes. The precise nature of this relationship is complex and it appears to depend on a number of variables, including the parent colony of the bee, the age (or developmental stage) of the bee and the amount of DWV present (or stage of DWV infection). The literature indicates that the expression of these developmental genes is highly dynamic, even in bees unchallenged by *Varroa* or disease (Walldorf, *et al.*, 2000; Bomtorin, *et al.*, 2012; Mello, *et al.*, 2014), which makes the testing and interpretation of how DWV infection affects the programmed development in honey bees very challenging. However, this study suggests that the effect of DWV on bee development could be understood provided that key variables are identified and taken into account when designing experiments. In order to reveal how DWV infection is subtly affecting developmental gene expression in honey bees it is crucial to avoid

pooling bee samples, to closely control the developmental stage of the sampled bees, to factor in the bee genotype or genetic pool, and to closely control the dose of DWV.

Having designed experiments to closely control for the above factors, it is clear that DWV infection can alter the expression patterns of key developmental genes in infected honey bee pupae as they undergo morphological development to the adult life stage. This experimental system could be combined with the knowledge acquired in the previous two results chapters of this work to reveal more about this previously unknown association. For example, as well as being temporally determined, the expression of ecdysone-regulated genomic networks (Li and White, 2003) and Hox gene expression (Mallo and Alonso, 2013) is known to be tissue specific in insects. With our knowledge of the tissues most affected by DWV infection, both in terms of viral load and of developmental abnormalities in infected bees, it would be possible to dissect out particular organs or tissues (for example the brain, salivary glands, midgut epithelium or developing wing buds) and analyse developmental gene expression in these alone. This would further reduce any ‘background noise’ that is caused by testing the whole bee, including any organs or tissues less affected by DWV infection. It is also possible to study the transient expression of Hox gene expression using RNA-FISH (Bantignies and Cavalli, 2014), so it would be interesting to attempt to visualise and co-localise developmental gene expression and virus replication in honey bee tissue using our RNA-FISH and confocal microscopy techniques. This has potential to provide a mechanistic understanding of how DWV causes developmental abnormalities in virus infected bees.

When taken as a whole, the work described in this thesis addresses key gaps in the knowledge about *Varroa*/DWV interactions in honey bees, from the population level, down to the individual. The dynamics of early stage DWV infection in individual bees have been explored, both in terms of infectious doses and time coursed of infection for contrasting modes of transmission, and in terms of the effect on the developmental transcriptome of infected bees. The link between DWV infection and developmental

and cognitive abnormalities has been clear for some time (de Miranda and Genersch, 2010; Moeckel, *et al.*, 2011) but the results described in Chapter 5 represent, to our knowledge, the first published account of a possible mechanism for this pathology.

On a larger whole colony and population scale, this work addresses the conflicts in the published literature concerning the impact of *Varroa* mediated transmission of DWV on the viral diversity and potential selection of a virulent strain. As well as showing that DWV-like viruses dominate in recently *Varroa* colonised apiaries in an isolated area of Western Scotland, we have shown that there is likely to be a stable ‘climax population’ of a recombinant strain of the virus in our experimental Warwickshire apiary. These results, when considered alongside the results published by other research groups (Martin, *et al.*, 2012; Ryabov, *et al.*, 2014; McMahon, *et al.*, 2016b; Mordecai, *et al.*, 2016; Brettell, *et al.*, 2017), suggest a potential model for DWV strain selection, with early *Varroa* infestation appearing to select for DWV-like viral strains, with the possibility of further selection pressures causing a shift towards more VDV-like or recombinant strains as the *Varroa* mites become more established within an area. Further work is required, including continued environmental sampling and molecular analysis of DWV strains, and experimental manipulation of a reverse genetic system, in order to test this theory and reveal the possible mechanism behind this strain selection.

There are a number of original questions raised by the findings described in this thesis, and these have been briefly mentioned above. Here I will expand upon two of them and propose potential experiments to build upon the knowledge we have already acquired:

The potential role played by developmental gene expression perturbation during DWV infection in honey bees is an interesting and novel area of research. The results of my experiments indicate that virus replication may result in subtle alterations in the developmental gene transcriptome but further investigation is required before we could confidently conclude that these transient, variable disturbances are the previously unknown mechanism behind the pathology of DWV. In order to reveal more about this

delicate system, we must firstly find out more about the variability in the observed transcriptional responses of honey bees, and how this is linked to the bee genome and to the susceptibility to DWV infection.

I propose a larger scale multi colony injection experiment, injecting geographically (and therefore genetically) distant bees with a controlled dose of DWV. The results of this experiment could be linked to a DWV dose response or susceptibility experiment, to establish whether bees exhibiting certain perturbations to developmental genes are more likely to go on to develop symptomatic DWV infections. It would also be interesting to learn more about the genotypes of the experimental colonies, perhaps using a single nucleotide polymorphism (SNP) based assay, as described by (Muñoz, *et al.*, 2015; Henriques, *et al.*, 2018) to establish if the between colony variation in response is determined by honey bee genotype.

Experimental manipulation of the expression of Hox and ecdysone responsive genes in developing pupae would give a clearer indication of the impact that disturbances to these pathways has on honey bee physiological or cognitive development. Sub lethal effects on behavioural reflexes could be measured by a proboscis extension response assay (Iqbal and Mueller, 2007; Smith and Burden, 2014). This manipulation could be achieved through RNAi-mediated gene knockdown, a technique which has been shown to be effective in suppression of developmental and regulatory gene expression in adult honey bees (Corona, *et al.*, 2007; Ament, *et al.*, 2012; Wang, *et al.*, 2013). Experimental manipulation of developmental gene expression could be usefully combined with the RNA-FISH techniques developed in Chapter 3 of this work, to reveal more about the tissue specificity and association with DWV infected tissues of the candidate genes.

There is also further work to be done in determining why *Varroa* mediated transmission of DWV selects for certain strains of the virus, and why these strains appear to differ according to the geographical location or stage of *Varroa* colonisation of the sampled bees. I propose a longer-term sampling experiment in Ardnamurchan, to establish if the shift towards DWV-like strains is stable or if the predominant strain changes after further

years of *Varroa* parasitism. I also think that next generation sequencing of global samples should be conducted to ascertain the dominant strain of DWV in all the areas where *Varroa* mites have become established. Alignment of these global full length DWV sequences would allow identification of any conserved elements of the genome, potential candidates for conferring this selective advantage.

A reverse genetic system, which is now possible following the identification and publication of an infectious full length DWV clone (Lamp, *et al.*, 2016), would allow testing of viral clones containing any candidate sequences identified in the global sequencing programme. Identification of the virulent strains of DWV, or of the element of the DWV genome that confers a selective advantage during *Varroa* transmission, would shed more light on why the DWV/*Varroa* combination has proved so deleterious to honey bee health worldwide. It may also provide potential targets for developing anti-viral treatments.

The results of this work have implications, not only in informing future research and resolving key gaps in the knowledge about *Varroa*/DWV interactions in honey bees, but also on a practical level for beekeepers. DWV is an extremely harmful pathogen of honey bees, replicating freely within many tissue types and quickly causing symptomatic infections in *Varroa* exposed individuals, and strategies to prevent its accumulation within colonies would be beneficial for honey bee health worldwide.

Traditionally beekeepers in *Varroa* afflicted areas treat their colonies to remove the mites at the end of the season, after honey harvesting in September, to remove the mites before the over winter worker bees are produced and the winter break in brood production and foraging occurs. However, the results of this work indicate that, by September, unchecked *Varroa* levels and DWV load are likely to have already risen to harmful levels, as seen in our single apiary experiment (Chapter 4 – section 4.3.2). We have also shown how quickly DWV infections can progress in infected bees and how the transition to *Varroa* mediated transmission can alter the prevalent strain of DWV within a colony, potentially shifting towards more pathogenic strains. In a late season colony where the majority of

bees are exposed to DWV, either via *Varroa* parasitism or in high does orally through hygienic behaviour (cannibalism of DWV infected brood), there are likely to be serious negative consequences for the crucial over winter workers, including perturbations to the expression of developmental genes. The results of this thesis serve to highlight the importance of early and consistent *Varroa* control strategies. The National Bee Unit (BeeBase, Fera) publish an excellent guide to *Varroa* management (<http://www.nationalbeeunit.com/downloadDocument.cfm?id=16>) which includes strategies on integrated pest management for *Varroa* control. These focus on biotechnical methods early in the season (such as drone brood removal) and varroacides (such as thymol or oxalic acid) later in the season after the honey has been collected or the brood rearing season has finished. The results of this thesis may inform future advice for bee keepers, perhaps increasing the focus on early season control methods.

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Appendix 1 – Table of oligonucleotide primers

Primer ID	Primer sequence (5' to 3')	Description
DWV-like-366	CTGTAGTTAAGCGGTTATTAGAA	qPCR-VDV-Struc-For
DWV-like-367	GGTGCTTCTGGAACAGCGGAA	qPCR-VDV-Struct-Rev
DWV-like-368	CTGTAGTCAAGCGGTTACTTGAG	qPCR-DWV-Struct-For
DWV-like-369	GGAGCTTCTGGAACGGCAGGT	qPCR-DWV-Struct-Rev
DWV-like-370	TTCATTAAAACCGCCAGGCTCT	qPCR-VDV-Pol-For
DWV-like-371	CAAGTTCAGGTCTCATCCCTCT	qPCR-VDV-pol-Rev
DWV-like-372	TTCATTAAAGCCACCTGGAACA	qPCR-DWV-pol-For
DWV-like-373	CAAGTTCGGGACGCATTCCACG	qPCR-DWV-pol-Rev
Bee-actin -374	AGGAATGGAAGCTTGCGGTA	qPCR-Bee-actin-For
Bee-actin -375	AATTTTCATGGTGGATGGTGC	qPCR-Bee-actin-Rev
Varroa-actin-132	TGAAGGTAGTCTCATGGATAC	qPCR-Varroa-actin-For
Varroa-actin-130	TGAAGGTAGTCTCATGGATAC	qPCR-Varroa-actin-Rev
DWV-like -163	GTTTGTATGAGGTTATACTTCAAGGAG	qPCR-Universal-DWV-like-pol-R
DWV-like -164	GCCATGCAATCCTTCAGTACCAGC	qPCR-Universal-DWV-like-pol-R
DWV-like-155	CAGTAGCTTGGGCGATTGTTTCG	Nested-outer-Uni-DWV-4700-For
DWV-like-156	CGCGCTTAACACACGCAAATTATC	Nested-outer-Uni-DWV-6700-Rev
DWV-like-151	CTGTAGTCAAGCGGTTACTTGAG	Nested-Inner-DWV-4900-For
DWV-like-152	CTGTAGTTAAGCGGTTATTAGAA	Nested-Inner-VDV-4900-For
DWV-like-153	CTTGGAGCTTGAGGCTCTACA	Nested-Inner-DWV-6500-Rev

DWV-like-154	CTGAAGTACTAATCTCTGAG	Nested-Inner-VDV-6300-Rev
DII S1 F	ACGCCTACGGATATCACCTG	Distal-less qPCR For
DII S1 R	CCCTTTACCGTTCCTCAAG	Distal-less qPCR Rev
Btnless S1 F	TGCCGAATATACAGAAGATGGCA	Buttonless qPCR For
Btnless S1 R	CGATGGAATCTCCCAAGCGT	Buttonless qPCR Rev
AbdA F	ACAACCACTACCTGACGCG	Abdominal A qPCR For
AbdA R	ACTCCTTCTTCAATTCATC	Abdominal A qPCR Rev
ExEx S1 F	TAAGGTGGAGGATGGTGGG	ExtraExtra qPCR For
ExEx S1 R	CGGTCGTGTTTCTTTGCGTG	ExtraExtra qPCR Rev
Drop1 S2 F	GCACCCTGAGGAAGCACAAG	Drop1 qPCR For
Drop1 S2 R	GGCGATGGTTAGGTACTGCT	Drop1 qPCR Rev
EcrA F	CCAACAGCAACAACGGCTAC	Ecdysone Receptor variant A For
EcrA R	AAAGAGCCAGGCTGCGACAA	Ecdysone Receptor variant A Rev
EcrB F	ACAGTGTTGCCAACGGTCAC	Ecdysone Receptor variant B For
EcrB R	AAAGAGCCAGGCTGCGACAA	Ecdysone Receptor variant B Rev
InR S2 F	CCACTGTCCTGCCGGTTATT	Insulin-like peptide receptor For
InR S2 R	ACGAATATAGATGGGCGGGG	Insulin-like peptide receptor Rev
Crol S2 F	ACGAAGCGAGATGCCAGAAC	Crooked legs For
Crol S2 R	CCCGCGTTTACATCCTCTTG	Crooked legs Rev

Appendix 2. Sequencing of central region of Ardnamurchan Samples

>2015Baker2.1.1

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>2015Baker2.1.2

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>v2015Baker2.1.4

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>2015Baker2.1.5

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>2015Baker2.2.1

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>2015Baker2.2.2

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>2015Baker2.3.4

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>2015Baker2.3.5

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>2015Baker2.1.6

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>2015Baker2.2.6

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>2015Baker2.3.6

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>2015Baker2.1.7

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>2015Baker2.2.7

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>VDV-1

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>DWV.Lanzi

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>SB2.2.1

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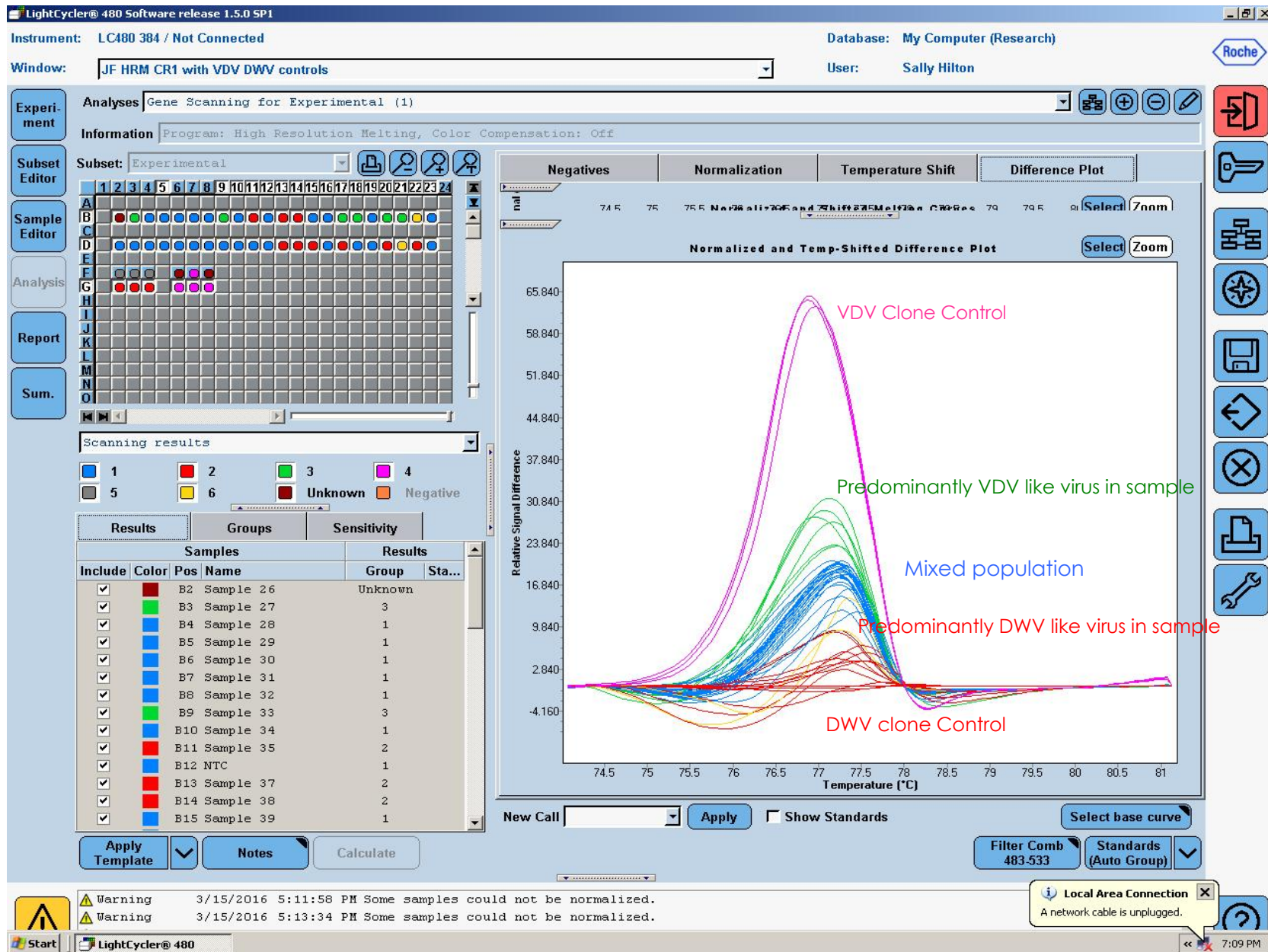
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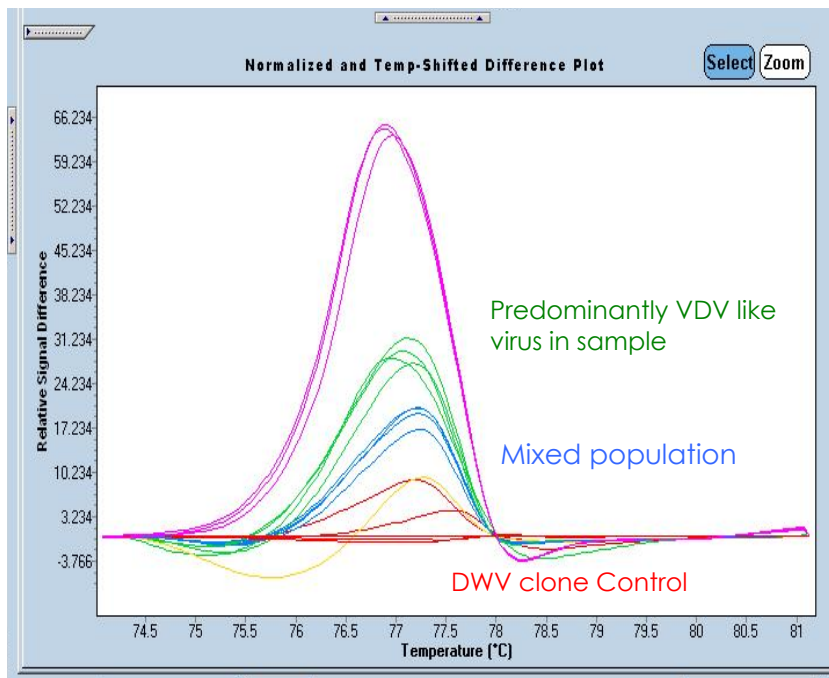
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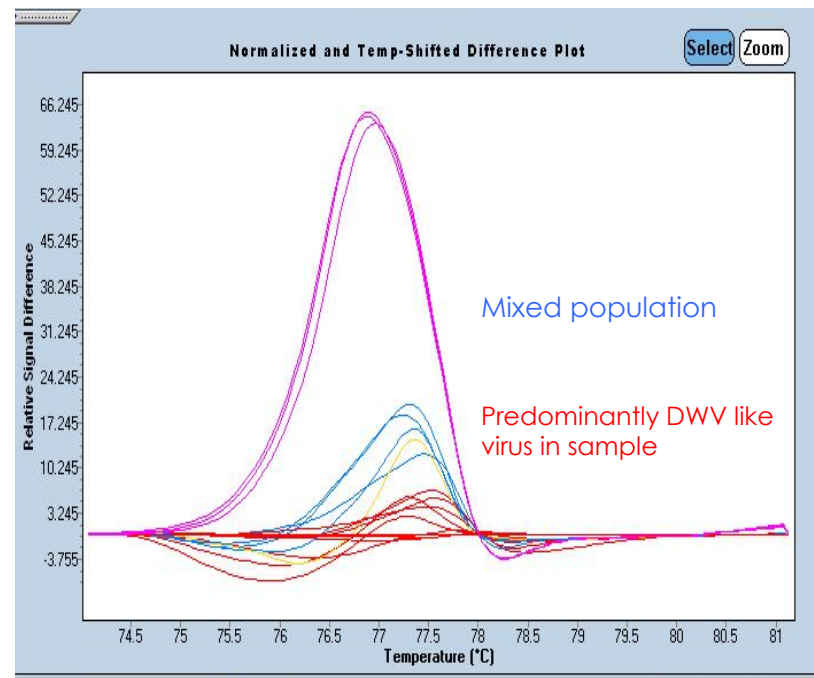
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Blue colony – Varroa infestation.

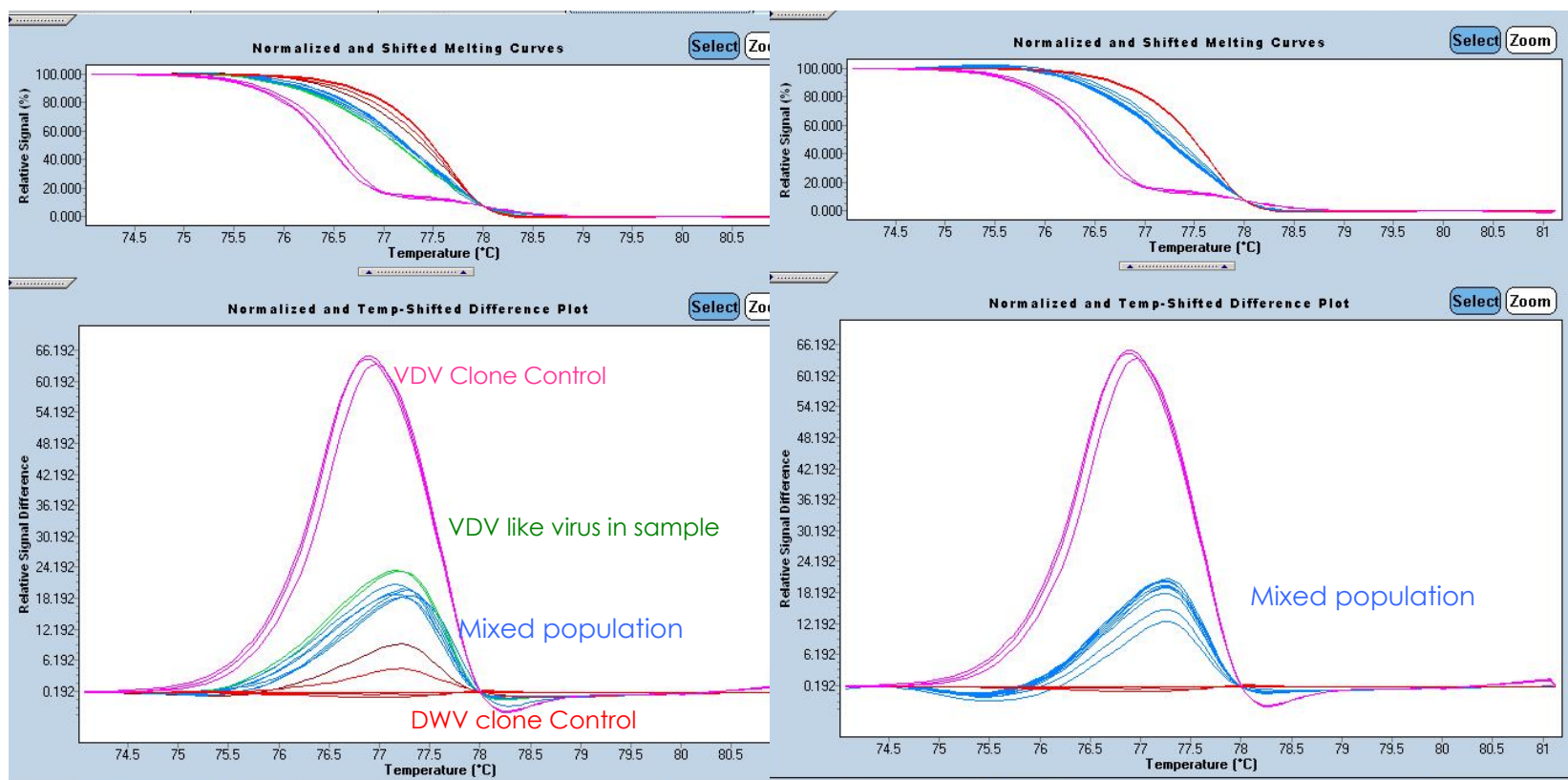


Blue 1 (start of season)



Blue 13 (end of season)

White Colony – Very low levels of Varroa



White 1 (start of season)

White 13 (end of season)