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Discovery of *Apis mellifera filamentous virus* in the blue orchard bee: Characterization of impacts and interactions with an organosilicone surfactant

Running head: AmFV in *O. lignaria* and interactions with OSS

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Abstract

While RNA viruses have received attention in impacts on bee health, research is limited for DNA viruses. DNA viruses, such as *Apis mellifera filamentous virus* (AmFV), have been identified in solitary bees; but infection dynamics and impacts are not well studied. We detected AmFV in diapausing *Osmia lignaria* adults and dead larvae from wild populations using metagenomic sequencing. Finding AmFV in diapausing adults in cocoons indicated an active AmFV infection. Unmodified larval provisions from nests had significant levels of AmFV, suggesting virus transmission may occur via fecal/oral routes via pollen. Using established rearing methods, we fed *O. lignaria* larvae AmFV and monitored survival, development, and adult weight metrics. Increased amounts of AmFV in larval provisions resulted in increased mortality. Co-exposure to an organosilicone surfactant (OSS) in provisions synergistically increased AmFV titers in larvae and adults, indicating that agrochemicals could affect viral infections. With increased development time from 2nd instar to pupation, bees of both sexes weighed more; however, male and female bees were impacted differently by AmFV and OSS. OSS plus AmFV exposure significantly increased the time to pupation in female bees. Male bees exposed to OSS weighed more. These results provide crucial insights into AmFV disease dynamics.

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Keywords: DNA virus, solitary, bee, adjuvant, interactions, infection

Introduction

Bees are vital to natural ecosystems and agriculture, providing essential services such as pollination [1,2]; and, almost 30% of the world's diet depends on pollination [3,4,5,6,7]. However, bees face several stressors such as habitat loss, variable weather, pesticide use, parasites, diseases, decreasing floral diversity, and interactions with introduced species [8,9,10,11,12]. Stressors can interact synergistically, leading to exacerbated negative effects on pollinator health that then contribute to significant losses in ecological and agricultural pollination services [13,9,14,15,11,16].

Viruses pose a specific threat to bee populations. Many viruses were once thought to be species-specific, but studies have demonstrated that bee viruses can infect multiple species and are transmitted from managed to wild bee populations and vice versa [17,18,19,20]. Solitary bees, including *Osmia bicornis* (L., Hymenoptera: Megachilidae) and *O. cornuta* (Latreille), have had numerous pathogens detected that were thought to be honey bee-specific (*Apis mellifera* L., Hymenoptera: Apidae); but, the impact of these pathogens on health and survival is not understood, especially in unmanaged solitary bee populations [21,22,23,24,20,25]. Disease dynamics in a field setting are rarely studied and present a complex relationship between wild and managed species, environmental stressors, nutrition quality, and human intervention [26,10,27,28,29,30].

Various studies demonstrate significant impacts of RNA viruses on honey bee colony loss when simultaneously exposed to several environmental stressors [31,21,32,33]. Most research concerning pesticide-pathogen interactions and their negative synergistic effects have focused on honey bees [34,35,11,36]. Virus and agrichemical interactions can lead to increased mortality, impaired detoxification mechanisms, and reduced immunocompetence [37,9,38,39,14,40]; yet, these interactions are not always straightforward and studies are lacking for non-*Apis* species [34,30]. Similarly, few studies have examined interactions between viruses and adjuvants [38,39], which are substances added to pesticide mixtures to enhance pesticidal properties and performance [41]. Little is known about the prevalence and pathogenicity of DNA viruses, including interactions with pesticides and pesticide aids such as adjuvants.

Apis mellifera filamentous virus (AmFV) is a DNA virus recently revealed to be a pathogen in bee populations worldwide with a complex history of discovery and classification. AmFV is a large double-stranded DNA virus having a filamentous trifold genome within a rod-shaped enveloped virion [42,43,44,45,46]. In 1976 and 1977, Clark discovered that the previously classified *Rickettsia*-like disease infecting honey bees was a large DNA virus [47,42,43,45,48]. While

AmFV's taxonomic classification remains ambiguous, it is related to other large DNA viruses in the families *Nudiviridae*, *Baculoviridae*, and *Hytrosaviridae* [43,45,49,50,51]. AmFV has been detected on every continent, primarily in bees (Supplementary Table S1), and occasionally in other arthropods (Supplementary Table S2). In the Americas, Cornman [50] utilized short-read sequences to describe three distinct AmFV lineages or clades that correlate to different honey bee strains or subspecies. One strain or clade of AmFV was consistent with the introduction of Africanized honey bees (AHB, *A. m. scutellata* x *A. mellifera*) in Brazil during the 1950s. There is a need to investigate AmFV's impact on bee populations from different ecosystems.

In honey bees, AmFV infections are associated with weak colonies, presence of crawling or dead adult bees, detections in fat bodies, and can be associated with some RNA viruses and *Nosema apis* (Zander) (Microsporidia: Nosematidae) [42,52,53,54,55]. Milky white hemolymph is a unique characteristic of AmFV infection in honey bees that is not observed in other viral infections [47,42,43]. Some studies suggest AmFV has low virulence and prevalence, does not cause overt symptoms, produces rare acute infections, and is not associated with colony loss [52,56,53,48,54,57,58,55]. However, the original studies by Clark [47,42] found the virus in sick colonies in multiple states and countries. AmFV prevalence varies seasonally and geographically, where some studies find higher prevalence in spring or overwintering bees, and higher abundance in warmer, drier areas [54,59,60]. Transmission likely occurs vertically and horizontally, in a similar fashion to other bee-associated viruses [53,45,61]. Interaction studies between AmFV and other bee health stressors like agrochemicals are lacking.

Via metagenomic sequencing, we discovered infections of AmFV in wild populations of *Osmia lignaria* Say (Hymenoptera: Megachilidae), taken from wilderness areas of the U.S. Forest Service lands in northern Utah and southern Idaho. The primary objective of our research was to investigate and assess the impact of AmFV on solitary bee health. *Osmia lignaria* Say is an important solitary bee used in orchard and berry crop pollination and is increasingly managed in the U.S. [62,63,64,65,66,67,68]. We investigated AmFV prevalence in managed *O. lignaria*-collected provisions, and documented AmFV prevalence in various *O. lignaria* life stages from wild and managed populations. In rearing assays, we utilized *O. lignaria* laboratory rearing methods to manipulate *O. lignaria* larval diets to incorporate the virus and agrochemicals [69,70]. We investigated the impacts of feeding larvae AmFV when incorporated into larval food by itself and with co-exposure to an adjuvant organosilicone surfactant (OSS). We recorded survival,

development time, and adult weight metrics to investigate AmFV infection dynamics in *O. lignaria*.

Results

Was AmFV discovered in O. lignaria*, provisions, and inoculates?*

From nests collected in northern Utah and southern Idaho on US Forest Service lands (with no known exposure to honey bees), AmFV sequences were found in DNA extracts of pupae, diapausing adult females, and a combined sample of larvae and an adult female found dead in the nests via metagenomic sequencing and analyses using BlobTools (Supplementary Table S5). AmFV was also detected in a living, diapausing adult female that was cut out of her cocoon indicating that this was an active viral infection, since all gut contents had been purged prior to cocoon completion and pupation [80].

The 27 AmFV sequences (150 bp each) from dead larvae and an adult female were highly similar to AmFV (Supplementary Tables S5, S6), without any other similarity to other viruses or other organisms in the Core NT data base of NCBI. The percent sequence identity ranged from 80 to 100% identity for the 27 sequences. In comparison to the AmFV clades found by Cornman [50], three sequences had 100% identity to all the AmFV clades. For the other sequences, the highest degree of similarity for the majority (20/27) was to Clade 1 (reference sequence NC_027925.1), with 6/27 sequences having greater similarity to Clade 2, and no sequences with greater similarity to Clade 3 (Supplementary Table S6).

Detectable levels of AmFV were found in undisturbed and control sham-inoculated provisions from all collected nests from 2020-2022 (Figure 1a) and newly-sampled natural nests from gardens at the USDA/ARS Pollinating Insects Research Unit (PIRU) in Logan, Utah in 2024, with variable amounts detected in the provisions from the grafting events and between years as measured by qPCR for AmFV (Supplementary Table S7). Therefore, control treatments were considered as a "baseline" exposure in all analyses. Finding AmFV in the undisturbed provisions indicated that the virus can be transmitted via pollen or nectar, possibly through an oral-fecal route. Two ssRNA viruses were detected in some undisturbed provisions (Black queen cell virus (BQCV) in 21/24 provisions; Sacbrood virus (SBV) in 4/24 provisions) (Figures 1b, 1c; Supplementary Table S7). The provisions with added viral inoculum did have higher AmFV titers and BQCV and SBV since these viruses were in inoculum add to these treatments. No

Deformed wing virus (DWV) nor Israeli acute paralysis virus (IAPV) were detected in the undisturbed provisions [78]. Some provisions inoculated with virus did have detectable IAPV (not shown), since IAPV was also in the viral inoculum.

Does added exposure of AmFV impact* O. lignaria *survival?

Survival from 2nd instar to pupation was significantly different between bees fed different concentrations of the AmFV inoculate (Parametric Survival, DF = 3, Wald χ^2=23.703, P<0.0001) (Figure 2). Bees exposed to the lowest inoculate concentration had the highest survival, and bees exposed to the highest inoculate concentration had the lowest survival, with survival for all added virus concentrations significantly different from the control provisions (baseline) (Generalized Regression with Normal Lasso with AICc Validation (DF = 2, Wald χ^2=45.321, P<0.001), multiple comparisons using Fisher's LSD pairwise comparisons [P< 0.05]).

Survival from 2nd instar to emergence was significantly different between bees fed different concentrations of the inoculate (Parametric Survival, DF=3, χ^2=13.125, P=0.004) (Figure 2). Like the 2nd instar to pupation survival model, the lowest concentration had the highest survival, and the highest concentration group had the lowest survival (Generalized Regression with Normal Lasso with AICc Validation (DF = 3, Wald χ^2=9.78, P=0.021), multiple comparisons using Fisher's LSD pairwise comparisons [P< 0.05]) (Figure 2).

Most of the death occurred in the larval stage as compared to pupae and adults (Figure 2). Figure 3 presents examples of dead larvae that failed to molt into pupae. Fewer adults successfully chewed out of their cocoons when exposed to the highest inoculate concentration compared to other treatments, with death occurring after adult molt and during diapause.

How do AmFV detections vary across* O. lignaria *life stages and wild populations?

Larvae had a higher prevalence of AmFV than prepupae, pupae, and adults reared in laboratory studies (two-sided Fisher's Exact test: P<0.01) (Table 3).

We found AmFV in wild *O. lignaria*. Although not significantly different, wild *O. lignaria* had a higher prevalence of AmFV at the Jardine Juniper site in Logan Canyon compared to other locations around Cache County, Utah, and the Cub River site in Idaho (two-sided Fisher's Exact test: P=0.31) (Table 4). The prevalence of AmFV in adults was lower as compared to immature life stages, mirroring results from the rearing assays (two-sided Fisher's Exact test: P<0.01) (Table 3).

Does added exposure to organosilicone spray adjuvants (OSS) affect AmFV infections and development?

When larvae ingested OSS, the relative titers of AmFV increased as the dosage of OSS increased (Linear regression, $F(1,155)=8.85$, $P=0.003$), with exposure to 100 and 500 ppm resulting in significantly higher titers (ANOVA, DF=197, $F=4.927$, $P=0.008$, Fisher's LSD post-hoc analysis). In emerged adults that had eaten OSS and AmFV as larvae, this increased titer of AmFV persisted in some adults (linear regression, $F(1,146)=6.08$, $P=0.015$). Adults exposed to 500 ppm as larvae had significantly greater AmFV titers than those eating no or 100 ppm OSS (ANOVA, DF=143, $F=3.198$, $P=0.044$, Fisher's LSD post-hoc analysis) (Figure 4).

How do AmFV and OSS affect developmental timing and post-diapause weight in emerged adults?

The distribution of adults that successfully emerged was not evenly distributed between males (n=325) and females (n=102). For the grafting of eggs, all available eggs from each nest were used, which may have provided a greater proportion of male eggs vs. female eggs [81,65].

To ask how AmFV and OSS affected development, we needed to consider the two ways in which larvae were exposed to AmFV (by AmFV in the provision itself and through added AmFV inoculum via the virus treatments) and then ask if OSS affected this interaction.

For development timing from 2nd instar molt to pupation, data from emerged females and males were considered. For females, there were interactions between the amount of AmFV in the larval provisions, exposure to OSS and added virus (Figures 5). When exposed to provisions alone having different AmFV amounts or with added AmFV, there was no significant change in the days needed to develop from 2nd instars to pupae for females. OSS impacts on development were dependent upon the amount of AmFV. When only OSS was added to the provision, the development time from 2nd instar to pupation for females was shortened as the amount of AmFV in provision increased (Regression, $F(1,21)=10.27$, $P=0.004$). With addition of both virus and OSS to the provision, the development time significantly increased as the amount of AmFV in the provision increased (Regression, $F1,27)=14.03$, $P=0.001$). For males, development time was not impacted by amount of AmFV in the provision nor by added OSS. There was a trend towards shorter development time for males if provisions had added AmFV as the amount of AmFV in the provision increased (Regression $F(1,79)=3.68$, $P=0.059$).

The post-diapause weight of bees was dependent upon sex, with female bees weighing significantly more than males (2 -way ANOVA, DF=389, F ratio=43.786, P<0.0001; Sex, DF=1, F ratio=84.029, P<0.0001) (Fishers LSD, P=0.05) (Figure 6). The development time from 2nd instar to pupation differentially affected the post-diapause weight of males versus females. Males that took longer to develop weighed significantly more (Regression, F(1,182)=6.36, P=0.013) and exposure to OSS in the larval provision resulted in a significant increase in post-diapause weight of males only (2-way ANOVA, DF=310, F ratio=16.378, P<0.0001; relationship to development time from 2nd instar to pupation, DF=1, F ratio=14.267, P=0.0002; OSS exposure, DF=1, F ratio=13.497, P=0.0003). For females, there was no impact on post-diapause weight by ingestion of OSS (Figure 6). For females only, inclusion of added AmFV in provisions interacted with development time from 2nd instar to pupation to result in decreased post-diapause weight (Figure 7) (3-way ANOVA, DF=102, F ratio=3.0284, P= 0.033; relationship to development time from 2nd instar to pupation, DF=1, t ratio=2.59, P=0.011; added AmFV exposure, DF=1, t ratio=-1.14, P=0.257; interaction of development time and added AmFV, DF=1, t ratio=-2.48, P= 0.015) (Figure 7).

Discussion

Our studies address a critical knowledge gap concerning DNA viruses and their impact on solitary bees. We found AmFV in *O. lignaria* nesting in wild areas in northern Utah and southern Idaho, on U.S. Forest Service lands. Several of these areas are classified as wilderness and all have no known history of honey bee colonies. The viral infection was found in diapausing adult female bees, cut out of their cocoons, indicating that the AmFV was an active infection and not an environmental contaminant. The virus was also detected in dead larvae and dead adults from the nests. The partial sequence of the virus from these bees was most similar to Clade 1 sequences of AmFV, but some sequences were more similar to Clade 2 [50]. According to Cornman [50], most of the managed colonies owned by commercial beekeepers have Clade 2 AmFV. There were no sequences similar to the AmFV found in Africanized honey bees (Clade 3). Additional sequences from the AmFV in *O. lignaria* and other non-Apis species are needed to determine the evolutionary history of this virus and to fully understand the strains. Using PCR with primers for the Bron gene, AmFV has been detected in other non-*Apis* species of bees, wasps, and flies in Europe, Asia, and South America; but infections have not been confirmed nor

the impacts determined [18,82,83,59,84,85,86]. For vespid wasps in China, AmFV was detected in pupae, and molecular phylogeny of the amplified region of AmFV was similar to the strains found in honey bees [87].

All tested provisions from nests in apple orchards (undisturbed, baseline-control, and virus-inoculated) and new nests from the PIRU garden had detectable AmFV, indicating that the virus is transmitted via fecal contamination or from parent to offspring (vertically and/or horizontally). Our research supports that larvae can acquire AmFV through provision consumption, comparable to horizontal transmission of AmFV in honey bee colonies via consumption of contaminated honey and pollen [45] and royal jelly [61]. Whether *O. lignaria* mothers provide AmFV-contaminated nectar and pollen to offspring, mix AmFV from contaminated nectar in her crop, or by other secretions is unknown and requires further investigation. Likewise, how AmFV persists in the environment is unknown. Given the lack of honey bees in the wild land areas we sampled, we have no evidence that the AmFV in *O. lignaria* is a result of pathogen spill over from honey bees. Additional research is needed to determine the cross-species transmission of AmFV via contamination of pollen.

We demonstrated that increasing AmFV exposure impacts immature stages of *O. lignaria*, leading to early death and increased amounts of AmFV in larvae. When fed the highest concentration of AmFV, *O. lignaria* larvae experienced lower survival to pupation and emergence. Some bees dying as pupae or adults were also found to have AmFV infections.

Higher AmFV prevalence in larvae mirrors results found for BQCV and SBV in reared larvae exposed to ssRNA viruses [78]. Whether this increased prevalence is due to an active infection or reflects the virus present in the gut contents needs to be determined. As found in this study, AmFV infections can persist through development into the adult stage; whereas BQCV and SBV were lost at the larval-pupal molt in 100% of all reared bees of *O. lignaria* [78]. AmFV does produce a systemic infection in *O. lignaria* that can persist throughout metamorphosis.

The development of the adult bees was differently impacted by AmFV and OSS depending upon the sex of the bee. Interactions between AmFV in the larval provision, added AmFV inoculum, and exposure to OSS were found. Exposure to OSS in the larval provision and increasing amounts of AmFV in the inoculum and provision resulted in longer development timing from 2nd instar to pupation for female bees, while exposure to OSS alone and AmFV in

the provision resulted in shorter development time. Male development timing tended to be shorter with increased AmFV.

The weight of newly-emerged male bees was impacted by both time required for development from 2nd instar to pupation and by OSS. Longer development time and exposure to OSS both resulted in increased male weight. This response to the organosilicone spray adjuvant is different than the response to the active ingredient deltamethrin (technical grade), where exposure resulted in decreased weight in male *O. cornifrons* and impacts on reproductive functions [88]. Additional research is needed to determine how other pesticides and formulated products applied to orchards impact development in *Osmia* species, given their use as pollinators in commercial crops [89].

While pathogen prevalence during pollination events has been well studied in honey bees, AmFV prevalence throughout the pollination season in the U.S., especially in managed solitary bees like *O. lignaria*, remains unexplored [90,91,92,93]. Adult *O. lignaria* live a few weeks after mating [65]; therefore, there is a short window to track AmFV levels in the environment during orchard pollination. Additionally, we documented that AmFV persisted through development into the adult stage (4.2% with AmFV). This finding indicates that AmFV produces a systemic infection in *O. lignaria* that can persist throughout metamorphosis. Investigating AmFV's persistence in adults as they age will help determine if its levels are higher shortly after adult *O. lignaria* emergence and if infectivity decreases over several days or weeks.

In this publication, we provide a step forward in understanding how AmFV impacts solitary bees while providing a path for future pollinator pathology research. As *O. lignaria* and other *Osmia* species become increasingly managed for pollination in the U.S., understanding AmFV transmission dynamics becomes crucial. Future studies will inform and improve *O. lignaria* production, management practices, and health of wild and managed populations. The methodologies and findings from our studies can serve as a springboard for investigating AmFV prevalence, virulence, and pathogenicity in other solitary bee species, which contribute to a broader understanding of pathogen ecology in diverse pollinator communities. Subsequent studies can build a foundation for effective pollinator management strategies and population conservation against viral threats. As AmFV research continues, understanding intricate relationships between viruses and their hosts can ensure the health and stability of crucial pollinators in natural and agricultural ecosystems.

Materials and methods

Wild O. lignaria *populations*

For a molecular survey, wild *O. lignaria* were collected from four locations: Cub River, Birch Creek, Jardine Juniper, and Mendon (Supplementary Table S3). Bees collected from Birch Creek and Cub River were nest-trapped in 2018 using nest blocks with drilled holes (diameter ≈ 8 millimeters [mm]) lined with straws. In the fall, adult females were excised from cocoons and frozen immediately at -80°C. Bees from Jardine Juniper were collected using nest boxes housing bundles of cardboard tubes with straw inserts. Bees from Mendon were collected using nest blocks with 40 holes of mixed sizes (diameters = 4, 6, 8, and 10 mm) in 2018. Only confirmed *O. lignaria* were used from the Mendon site. Bees in cocoons were removed and allowed to undergo winter diapause until the following spring. Dead larvae and pupae from *O. lignaria* nests and emerged adults were frozen at -80°C.

Experimental rearing studies

In 2020 to 2022, *O. lignaria* adult bees were purchased from a Utah supplier (Mountain West Mason Bees, Riverton, Utah, U.S.) and allowed to create nests at an apple orchard in Logan, Utah (2 hectares [ha]) using established management practices [65,69,70]. The orchard is part of a fruit and tree farm offering other fruiting trees like peach, pear, and serviceberry, as well as flowering trees such as chokecherry, hawthorn, lilac, linden, and redbud. The orchard owners do not apply chemicals for pest management until *O. lignaria* nesting is complete. The apple orchard is surrounded by neighborhoods, potentially allowing *O. lignaria* to forage on alternate pollen and nectar resources. No known honey bee colonies were in the area.

Using quantitative PCR (qPCR), we discovered existing, natural AmFV in provisions. Leftover undisturbed and treated provisions were frozen immediately on the day of inoculation at -80°C and remained in storage until extraction. Provisions left over from virus-inoculated treatments had DNA extracted and sequenced. Samples (0.25 g) of undisturbed, control, and virus-inoculated provisions were extracted and analyzed by qPCR for AmFV as described below. Given that all provisions had AmFV, the control treatments are considered baseline exposure.

In 2020 to 2022, we investigated how the added AmFV inoculate and organosilicone surfactant (OSS) mixed into *O. lignaria*-collected provisions impacted survival, development, and adult weight metrics of lab-reared *O. lignaria*, using methods described in Williams et al.

[69,70]. Experiments asking how increasing exposure to AmFV affects development had four treatments: a control group (sham inoculate of sterile Ringer's Solution added to the provisions containing natural AmFV), a low concentration of added AmFV (low), a medium concentration of added AmFV (medium), and a high concentration of added AmFV (high) (Table 1). Ringer's Solution was made according to the recipe provided by Williams et al. [69]. For the different added amounts of AmFV, the high concentration of inoculate was diluted 10-fold to create the medium treatment and diluted 1:100 to create the low added AmFV treatment. Treatments were combined into *O. lignaria*-collected provisions as outlined in Williams et al. [69,70], then 0.35 g (± 0.01 g) of homogenized treated provision was provided to each egg or first instar larva.

OSS was chosen as a test chemical to examine agrichemical impacts on viral infections and resulting impacts on *O. lignaria* survival, development, and adult weight metrics. Treatments utilized in this study were: a control group (sham inoculate of sterile reverse osmosis [RO] water added to the provisions containing natural AmFV), an added AmFV inoculate exposure group (virus only, equivalent to the medium exposure treatment above), OSS exposure groups (100 parts per million [ppm] and 500 ppm), and a combination of OSS plus (+) AmFV inoculate (OSS+virus) for both 100 and 500 ppm (Table 1). The purchased Sylgard™ OFX-309 Fluid (DOW®, Indianapolis, Indiana, U.S.) had an average concentration of 80% (DOW® Sylgard™ OFX-309 Fluid safety data sheet, 2021), with a chemical make-up of 3-(3-hydroxypropyl)-heptamethyltrisiloxane, ethoxylated, acetate. Treatments were combined into *O. lignaria*-collected provisions [69,70] and 0.35 g (± 0.01 g) of homogenized treated provision was provided to each egg or first instar larva.

Pathogen inoculate

Honey bees were collected in 2017 from a hive in Newton, Utah. Ten honey bees were gathered from a hive where behavior was documented as "lethargic." Bees were homogenized with RO water, and the supernatant was either filtered through 415 filter paper (VWR International, 28320-041) to remove large bee debris and then centrifuged at 8,500 x g for 20 minutes and a ramping speed set to 2 (Sorvall® Biofuge primo R, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.). The pellet was washed four times repeatedly and then dissolved in RO water and aliquoted. Alternatively, the homogenate was filtered through a sterile 0.8 µm syringe filter and then a sterile 0.2 µm filter, and aliquoted. Aliquots were stored at -80°C. The inoculate was thawed at 4°C overnight for use.

342 ***DNA extractions***

343 For laboratory reared bees, DNA was extracted from whole bees using TRIzol™ reagent
344 (Invitrogen) and resuspended in 50 microliters (µL) of Tris EDTA (pH 8). DNA concentration
345 was determined using a Qubit® 3.0 Fluorometer (Invitrogen) and a double-stranded (ds) DNA
346 broad-range Qubit™ assay kit (Invitrogen) before use in qPCR. DNA was diluted 1:10 before
347 use in qPCR, and 1 µL of the dilution was used as template in qPCR. For wild-caught bees, DNA
348 was extracted from whole bees or adult abdomens. For provision extractions, 0.25 g of each
349 provision was extracted.

350 ***Discovery of AmFV in wild* O. lignaria*, provisions, and inoculates***

351 Genomic sequencing was performed at the USDA-ARS Tropical Pest Genetics and
352 Molecular Biology Research Unit (Hilo, Hawaii, U.S.) to detect pathogen sequences in *O.*
353 *lignaria*. DNA extracts were prepared for sequencing using an AVITI 2x150 Sequencing Kit
354 (high output, Element Biosciences 860-00001, San Diego, California, U.S.). Libraries were
355 sequenced using an Element AVITI System Sequencing Instrument (Element Biosciences). DNA
356 sequences were analyzed after removing adapter primer sequences and quality scores were
357 confirmed using BlobToolKit [71]. For each sample, sequence contigs were assembled and
358 assigned to their closest taxon using BLAST [72] and Diamond [73] using the nt database (NCBI,
359 https://ncbi.nlm.nih.gov). DNA samples included bees (larvae, pupae, and adults) from wild
360 nests, a combined sample of five dead larvae and adults from Mendon nests, the AmFV
361 inoculate, and virus-inoculated provisions from rearing studies. Individual sequence reads (150
362 base pairs [bp]) were mapped onto an AmFV reference genome using the Burrows-Wheeler
363 Alignment (BWA) software tool [74].

364 Sequences were aligned and compared to an AmFV reference genome (reference
365 sequence: NC_027925.1, [45] and assembled sequences from Short Read Archives (SRA) [50],
366 using NCBI's MegaBLAST tool [75]. For the pathogen inoculate, the number of unique reads for
367 AmFV were determined (Supplementary Table S4).

368 ***Determination of prevalence and relative amount of AmFV***

369 qPCR was conducted using a QuantStudio™ 6 Pro Real-Time PCR System (Applied
370 Biosystems™, Thermo Fisher Scientific) and analyzed with the Design & Analysis Software
371 (v.2.7.0, Thermo Fisher Scientific). The QuantStudio™ protocol was conducted using the
372 following parameters: holding stage at 95°C for three minutes followed by 36 cycles of 94°C for

15 seconds, 56℃ for 15 seconds, and 72℃ for 17 seconds. A melting curve step was included and consisted of three steps: 95℃ for 15 seconds, 55℃ for 45 seconds, and 90℃ for 15 seconds.

Each master mix reaction, one per primer set, included 5 µL of PowerUp™ SYBR™ Green Master Mix (A25742, Applied Biosystems), 0.2 µL of a forward primer (20 micromolar [µM]), 0.2 µL of a reverse primer (20 µM), and 3.6 µL of Ultra Pure™ distilled water (10977-015, Invitrogen). Reactions for AmFV used the primer set targeting the AmFV BroN gene region (Table 2). Primers for quantification of *O. lignaria* genomic DNA were designed for the single copy *Toll-like receptor 7* (*Toll-7*) gene (Table 2). Test sample reactions contained 1 µL of 1:10 DNA template (extraction described above). Each qPCR reaction was run with two positive controls and a negative control. The negative control lacked template DNA and used 1 µL of Ultra Pure™ distilled water. One positive control used *O. lignaria* genomic DNA to confirm successful amplification of the *Toll-7* gene. A second positive control contained AmFV DNA from honey bees to confirm the presence and successful amplification of AmFV. AmFV positive controls were created by extracting DNA from the inoculate used in the rearing studies then aliquoted and frozen at -80℃ until use.

The presence of AmFV was determined if a sample produced a quantification cycle value (Cq value) less than 36 during qPCR and if the sample melting temperature (T_m) matched that of the AmFV positive control T_m. Absence was determined if the sample did not produce a Cq value, was below the level of detection, or if sample T_m did not match AmFV positive control T_m. Detection of *Toll-7* was used as a measure of *O. lignaria* DNA within the sample and used to calculate relative abundance of AmFV in the bees by first normalizing AmFV Cq values to *O. lignaria Toll-7* Cq values to produce ΔCq values [76,38,77]. The relative amount of AmFV was then calculated as two to the negative ΔCq ($2^{-\Delta Cq}$). This final calculation was used for analyses. AmFV levels in provisions are reported as Cq values since a plant gene was not designed or included in reactions; equal amounts (0.25 g) of provisions were extracted as described above and 1 µL of 1:10 diluted DNA was used in reactions.

Single-stranded RNA (ssRNA) viruses in the provisions and bees were screened and measured using reverse transcription qPCR (RT-qPCR) [78]. Total RNA was extracted using TRIzol™ reagent (Invitrogen) and resuspended in 50 µL of Ultra Pure™ distilled water (Invitrogen). Concentration of total RNA was determined using a Qubit® 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts) and an RNA broad range

Qubit™ assay kit (Invitrogen, Thermo Fisher Scientific). cDNA was synthesized using a protocol modified from Cox-Foster et al. [79]. RNA was combined with random hexamers diluted to 0.07 micrograms (µg) per µL (20 µg stock, Promega, Madison, Wisconsin, US), dNTP mix (PCR grade) at 10 µM per nucleotide (Invitrogen, Thermo Fisher Scientific), 5X first strand buffer, 0.1 molar (M) of dithiothreitol (DTT, Invitrogen), and M-MLV reverse transcriptase (Promega; containing 200 units [U]), mixed thoroughly, and incubated for 10 minutes at 25°C, followed by an incubation at 37°C for 50 minutes, and one last cycle of 70°C for 15 minutes in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, California, US). The cDNA was used in qPCR reactions as described above to screen for bee-associated ssRNA viruses.

Statistical analyses

Statistical analyses were performed in JMP® Pro v.19.1.1 (JMP Statistical Discovery LLC, Cary, North Carolina, U.S.) with data visuals created in JMP. Before analysis, data distributions were analyzed for normality using a Shapiro-Wilk test.

For rearing assays, a Fisher's Exact test was used to determine if the number of bees positive for AmFV were different between life stages. For wild *O. lignaria*, a Fisher's Exact test was used to determine if the number of wild bee samples positive for AmFV was different between life stages and collection locations.

Survival; Exposure to variable concentrations of added AmFV inoculum

A parametric survival fit model with a Weibull distribution was used to evaluate survival from 2nd instar to pupation and 2nd instar to emergence. The amount of added AmFV was used as a predictor variable. Bees that died before the 2nd instar stage were excluded from survival analyses, since larvae begin feeding on provisions at the 2nd instar stage and handling may have impacted their development. Pupation was censored if the bee successfully pupated, and emergence was censored if an adult bee successfully chewed out of its cocoon. To account for adult death in survival analyses, an arbitrary "death date" was assigned as halfway during winter diapause for dead adults. A Wald test was performed simultaneously to determine survival differences between treatments.

A Fisher's Exact test was used to evaluate the number of deaths for different life stages (larvae, prepupae, pupae, and adults) between inoculate concentrations. Bees frozen prematurely for molecular analyses were excluded in this analysis.

Development timing and adult weight metrics as affected by variable concentrations of added AmFV

Development timing (number of days) was measured between 2nd instar to pupation. Development time analyses were assessed starting with the 2nd instar stage to standardize development and survival throughout the experiment, given that damage from the grafting itself could impact survival of eggs or first instars. For analyses of development from 2nd instar to pupation, bees that did not make it to the adult stage were excluded from analyses. Multi-factor ANOVA was used to determine the impacts of AmFV in the provisions, added AmFV inoculum, and OSS exposure on the time required to develop to pupation. Female and male bees were analyzed separately. *Post hoc* tests were performed using LSMeans Tukey's HSD tests or Fisher's LSD *t*-tests when models produced significant results ($P<0.05$).

Weight data were analyzed for post-diapause weight of emerged adults. Multi-factor ANOVA was used to determine the impacts of AmFV in the provisions, added AmFV inoculum, OSS exposure and the time required to develop to pupation, with female and male bees being analyzed separately. *Post hoc* tests were performed using LSMeans Tukey's HSD tests or Fisher's LSD *t*-tests when models produced significant results ($P<0.05$).

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Data Availability

Data are deposited in the USDA Ag-Data Commons (https://agdatacommons.nal.usda.gov/). For review purposes only, use this link: https://figshare.com/s/a7bcffeb2f8c1a8c2651. Once manuscript is accepted, the data will be made public.

Ethics declarations

Competing Interests: The authors have no competing interests to declare. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Figure Legends:

Figure 1. Comparison of the amount of detected (**A**) AmFV, (**B**) BQCV, and (**C**) SBV in provisions in treatments from separate grafting events (letters on X-axis) in 2020-2022. The detected amounts of viruses are indicated by Cq values from qPCR; a lower Cq number indicates a higher amount of detected virus. The colored ovals are treatments; the three provisions with different amount of added inoculum are highlighted (low, medium, and high) and outlined with a star. The grafting events were separated by at least one day from each other, with the nest provisions from each day pooled and homogenized to create the provisions used for the grafting

751 of the eggs/first instar larvae for the different treatments. All provisions had detectable AmFV,
752 and controls are considered to be a baseline treatment.
753
754 **Figure 2.** Survivorship of *O. lignaria* from second instar to pupation (top) and emergence
755 (bottom), when larval provisions have added amounts of AmFV [baseline (red), low (green),
756 medium (blue), and high (yellow)]. The x-axis represents the number of days from second instar
757 to pupation and emergence. The y-axis represents the proportion of bees surviving to pupation
758 and emergence respectively. Significant developmental time points are indicated on the figure.
759
760 **Figure 3.** Images of dead larvae fed different amounts of AmFV (baseline, low, and high
761 concentrations) in provisions. Larvae died prior to metamorphosis and failed to molt into pupae.
762 Scale bars are provided in millimeters (mm).
763
764 **Figure 4.** AmFV relative titers (AmFV/toll) increase when larvae are fed AmFV and OSS (100
765 or 500 ppm). Larval samples are in blue, with adult samples in red. The amounts of AmFV are
766 relative to the amount of toll DNA detected in the sample. The Y axis is exponential.
767
768 **Figure 5.** Impact of OSS and AmFV on time (days) needed to develop from a 2nd instar larva to
769 pupation, for males versus females. Amount of AmFV is reported as the total amount of AmFV
770 (determined using qPCR with Bron primers (Cq)) in the provision fed to the bees. A lower Cq
771 value indicates higher titers of AmFV. Treatments include Baseline (no added AmFV or OSS),
772 OSS (100 ppm added to provision), OSS+V (100 ppm OSS plus added AmFV inoculum), and

773 Virus (added AmFV inoculum). Sex was determined for each bee after adult emergence. A
774 regression analysis is reported for each treatment for both sexes.
775
776 **Figure 6.** Relationship between post-diapause weight of newly emerged adults versus the time to
777 develop from 2nd instar larvae to pupation for both males and females. Impact of OSS being
778 included in the provision (100 or 500 ppm OSS) is indicated by color of sample values and
779 regression lines (Blue= Controls, no OSS; Red= OSS at 100 or 500 ppm in provisions).
780 Regression lines are reported at top with a significant relationship between weight and
781 development time for males, but not for female bees. OSS resulted in increased weight of males.
782
783 **Figure 7.** For females only, the added AmFV in larval provisions interacted with days required
784 to develop from a 2nd instar larva to pupation. Blue samples and line = Control or Baseline
785 AmFV exposure. Red samples and line = Added AmFV in provision.
786
787
788
789
790

Tables:

Table 1. Treatment names and their corresponding combinations for each experiment. OSS was added as parts per million (ppm). Note for control treatments: AmFV was detected in provisions without adding the inoculate; therefore, we refer to control treatment groups as a baseline exposure.

Treatment name	OSS concentration	Added Virus	Description
Control (BASELINE)		No	Ringer's Solution alone
LOW conc.		Yes	1:100 dilution from HIGH treatment
MEDIUM conc.		Yes	1:10 dilution from HIGH treatment
HIGH conc.		Yes	Undiluted inoculate
Control (BASELINE)	0 ppm	No	RO water alone
Inoculate (INOC)	0 ppm	Yes	Inoculate alone
OSS	100 ppm	No	OSS at 100 ppm alone
	500 ppm	No	OSS at 500 ppm alone
OSS+INOC	100 ppm	Yes	OSS at 100 ppm + inoculate
	500 ppm	Yes	OSS at 500 ppm + inoculate

Table 2. Primers used in DNA PCRs to screen and quantify AmFV in samples. Primer sequences are provided in the 5' to 3' direction. T_m refers to the melting temperature of each primer in degrees Celsius. Amplicon size is reported in base pairs (bp). RNA virus primers are also presented in this table to screen provisions for ssRNA bee-associated viruses. The *Toll-like receptor 7* primer set was designed using the NCBI Accession XM_034315995.

Target or virus	Primer name	Primer sequence	T_m (°C)	Size (bp)	Reference
Toll-like receptor 7	q2Toll7-OL-F q2Toll7-OL-R	TGACCTTCCTGCCGTTACAC ACCACTTTGTGCGCATTGTC	59.97 59.97	193	This study
Apis mellifera filamentous virus	AmFV-BroN (F) AmFV-BroN (R)	TTATTAACACCGCAGGCTTC CATGGTGGCCAAGTCTTGCT	56.13 60.90	167	Hartmann et al., 2015
Black queen cell virus	BQCV 8195F BQCV 8265R	GGTGCGGGAGATGATATGGA GCCGTCTGAGATGCATGAATAC	59.02 59.52	71	Chantawannakul, 2006
Deformed wing virus	DWV Forward DWV Reverse	CTCGTCATTTTGTCCCGACT TTCATCAGGAGCACAACCTACAG	57.92 60.31	128	Singh et al., 2010 (F); Shutler, 2014 (R)
Israeli acute paralysis virus	IAPV-L17-GUS F IAPV-R130-GUS R	CGAACTTGGTGACTTGAAGG GCATCAGTCGTCTTCCAGGT	56.67 59.75	77	Cox-Foster et al., 2007
Kashmir bee virus	KBV-L34-GUS F KBV-R156-GUS R	CAAACTGCTGAATCAATGTCAAAAT ACATGCCTCTACTTTGTCACATTCA	57.58 60.75	123	Cox-Foster et al., 2007
Sacbrood virus	SBV Forward SBV Reverse	TGATGCAGTAGCTATGCGAGTGA GCGGCTCATCAGGATAAGCA	61.80 60.25	114	Levitt, Cox-Foster (unpub.); Fine et al., 2017

Table 3. Prevalence of AmFV in wild caught or laboratory reared bees by life stage. The percent (%) of bees positive for AmFV are presented followed by number (*n*) of bees that tested positive. Number of bees by life stage are presented to show distribution between tested life stages.

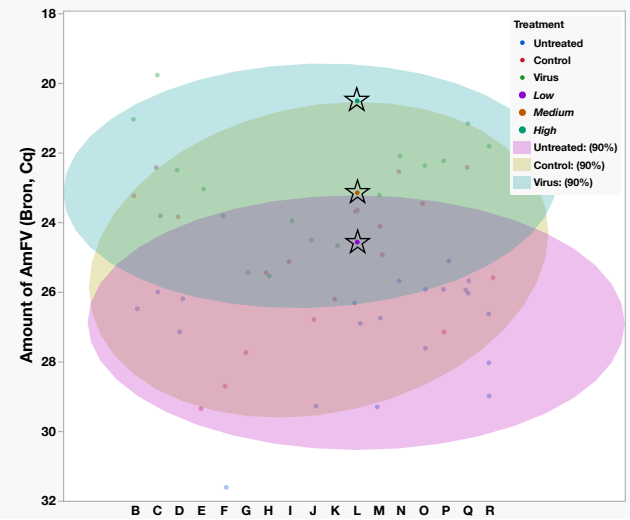
Category (*n*)	**AmFV (*n*)**
Wild caught bees (70)	*14 positive for AmFV*
Larvae (7)	42.9% (3)
Prepupae (3)	66.7% (2)
Pupae (6)	50.0% (3)
Adults (54)	11.1% (6)
Laboratory reared bees (460)	*190 positive for AmFV*
Larvae (188)	92.0% (173)
Prepupae (38)	23.7% (9)
Pupae (82)	1.2% (1)
Adults (152)	4.6% (7)

Table 4. Prevalence of AmFV in wild *O. lignaria* by location. The percent (%) of bees positive for AmFV are presented followed by number (*n*) of bees that tested positive. Number of bees by life stage are presented to show distribution between locations.

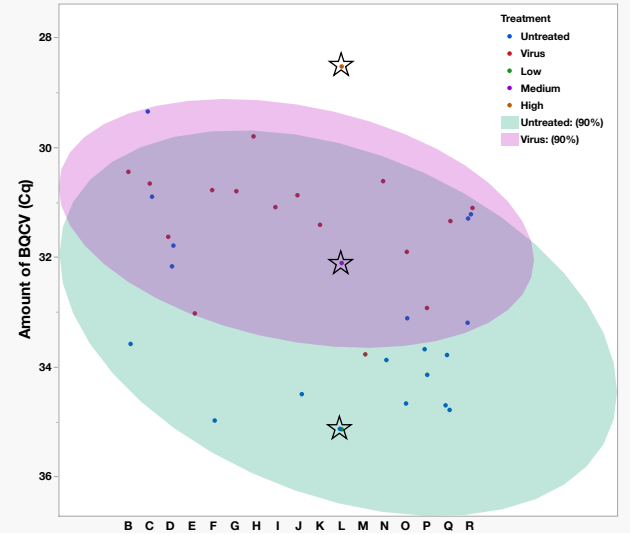
Location (*n*)	**AmFV (*n*)**	**Life stages surveyed**
Birch Creek (13)	7.7% (1)	Adults (13; 1 positive)
Cub River (5)	0% (0)	Adults (5; none positive)
Jardine Juniper (26)	30.8% (8)	Larvae (3; all positive) Prepupae (3; 2 positive) Pupae (5; 2 positive) Adults (15; 1 positive)
Mendon (26)	19.2% (5)	Larvae (4; none positive) Pupae (1; positive) Adults (21; 4 positive)

Figure 1, Williams, Schaeffer, Cox-Foster

A)



B)



C)

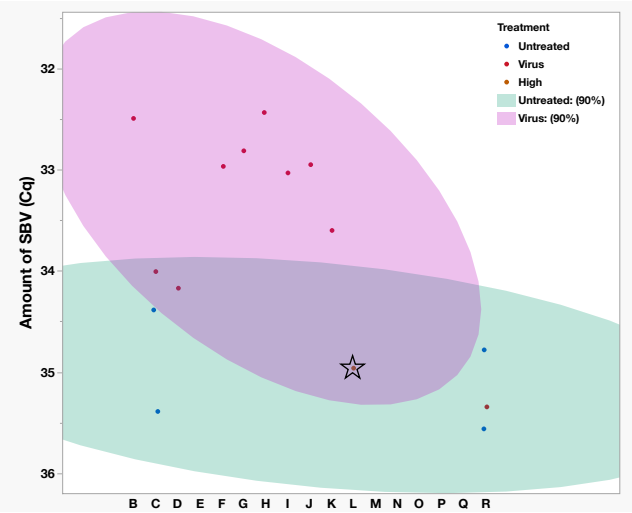
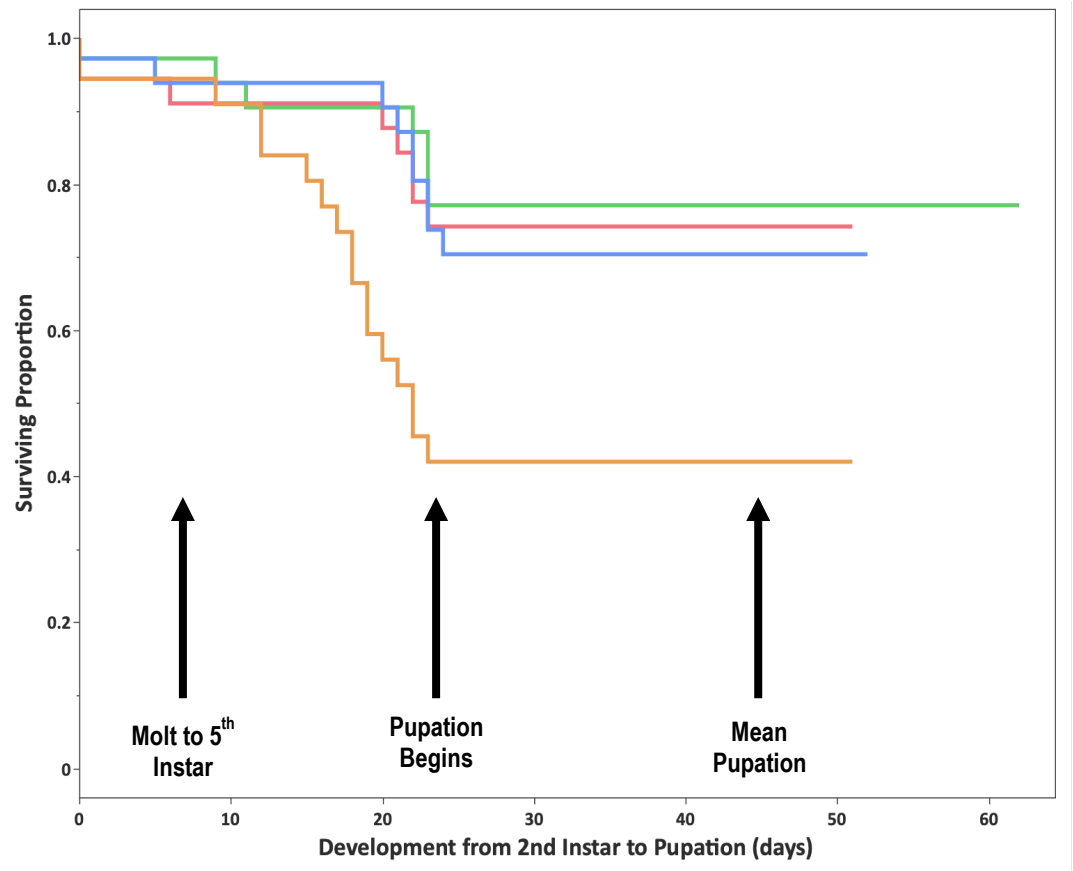


Figure 2. Williams, Schaeffer, and Cox-Foster



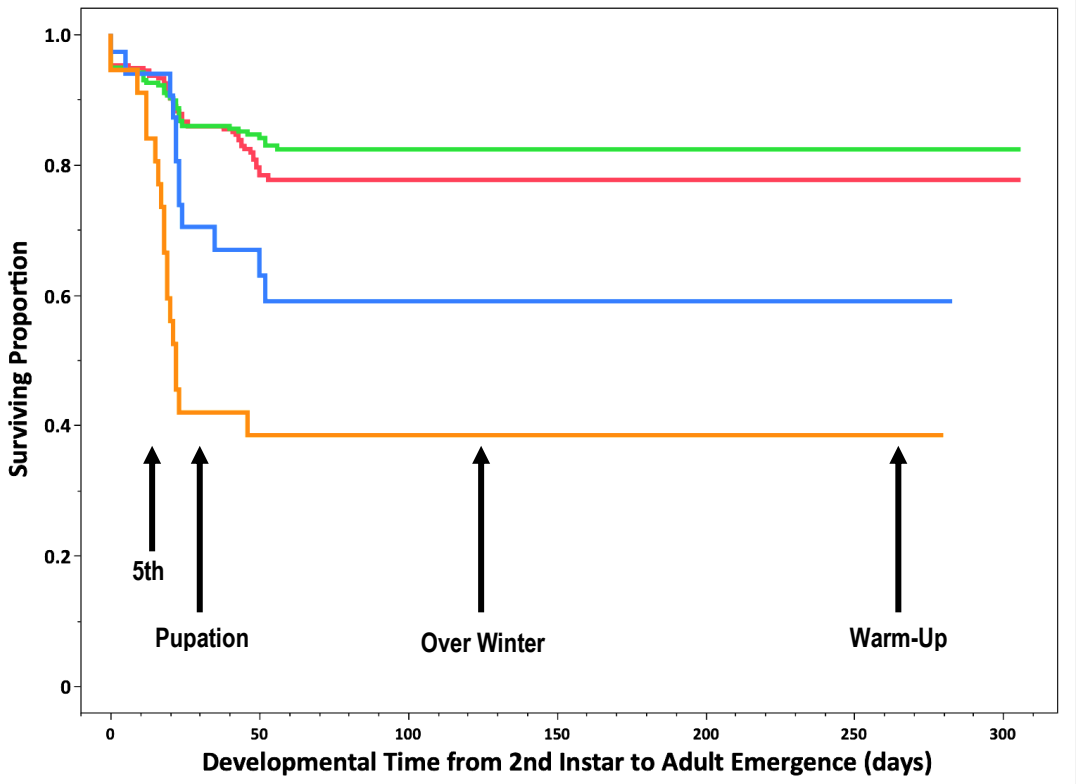


Figure 3, Williams, Schaeffer, and Cox-Foster

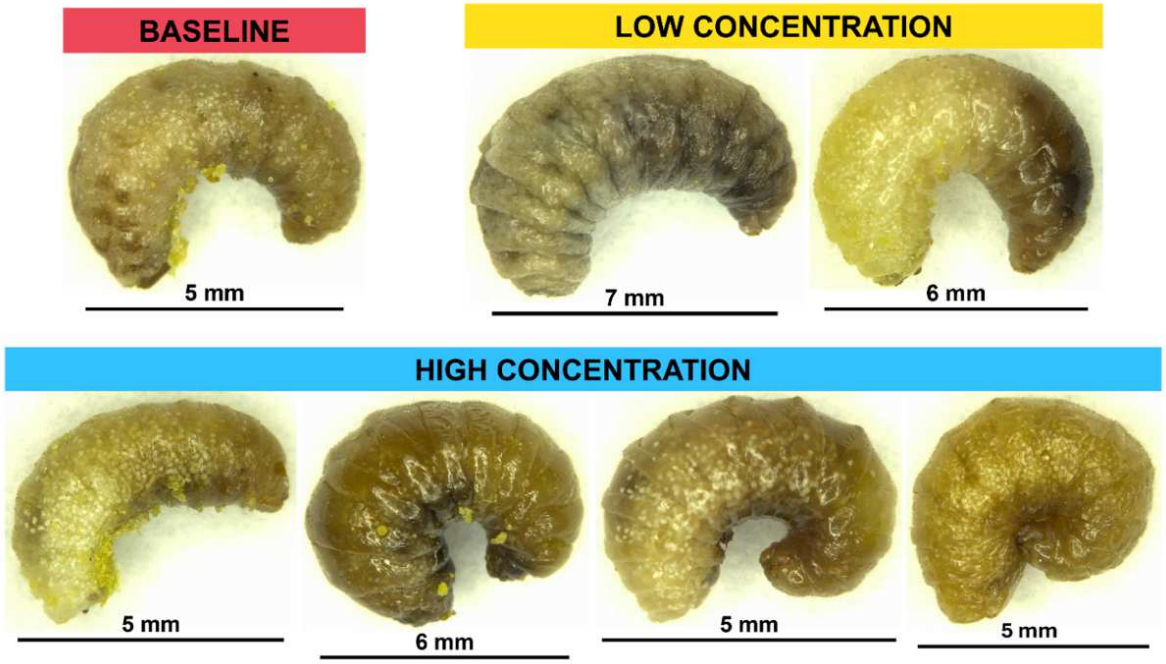


Figure 4, Williams, Schaeffer, and Cox-Foster

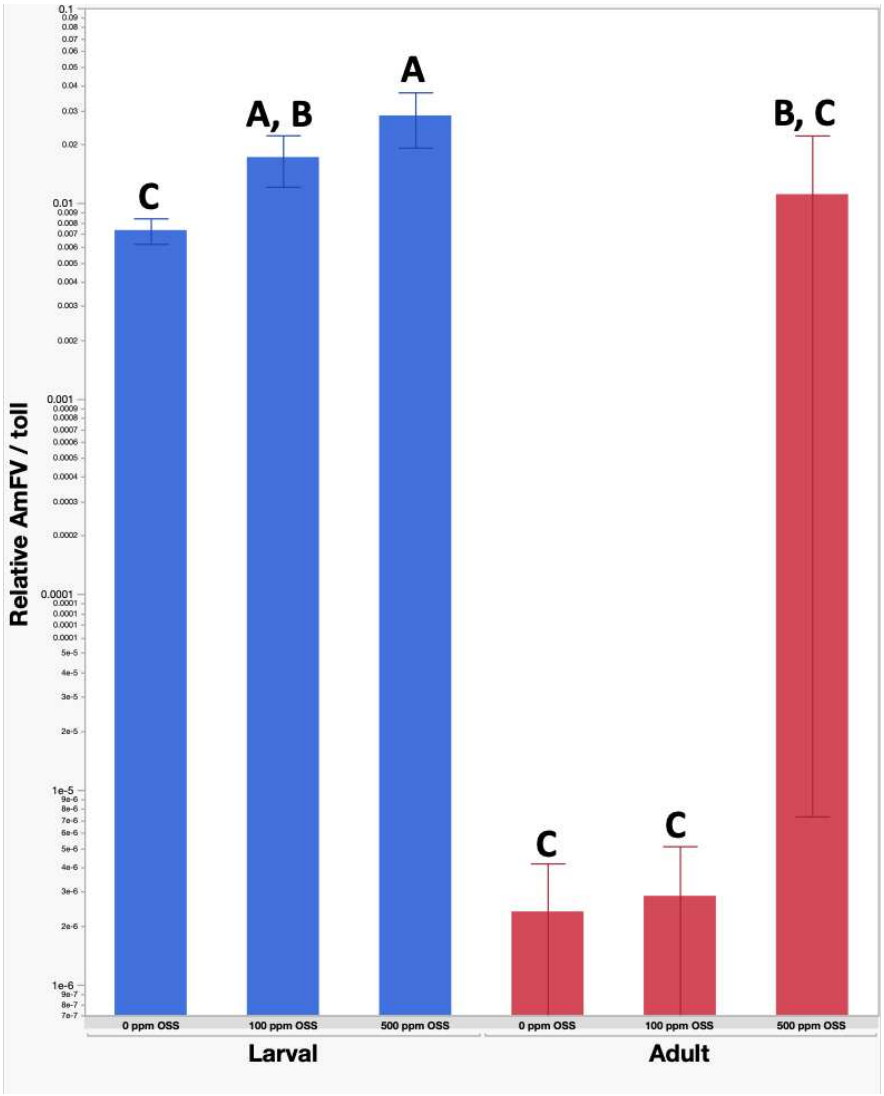


Figure 5, Williams, Schaeffer, and Cox-Foster

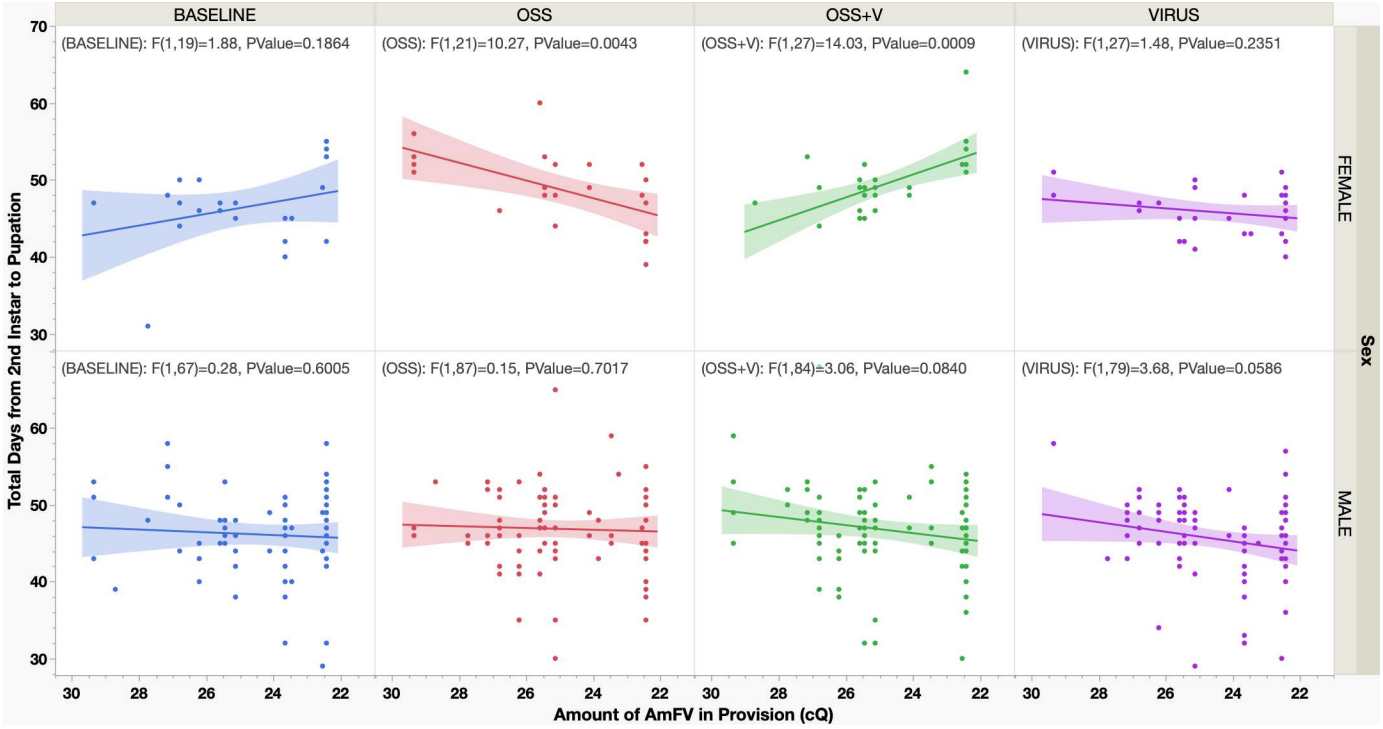


Figure 6, Williams, Schaeffer, and Cox-Foster

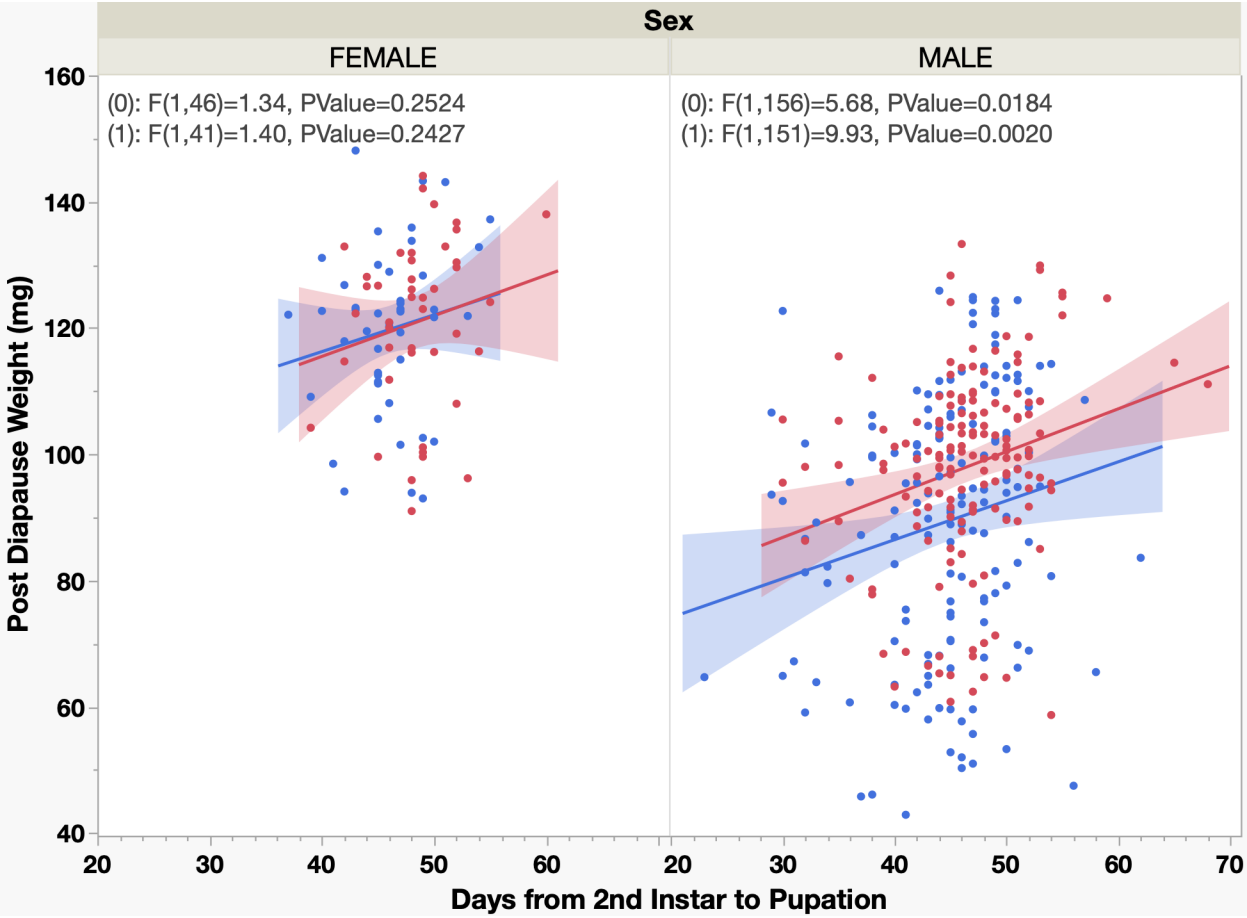
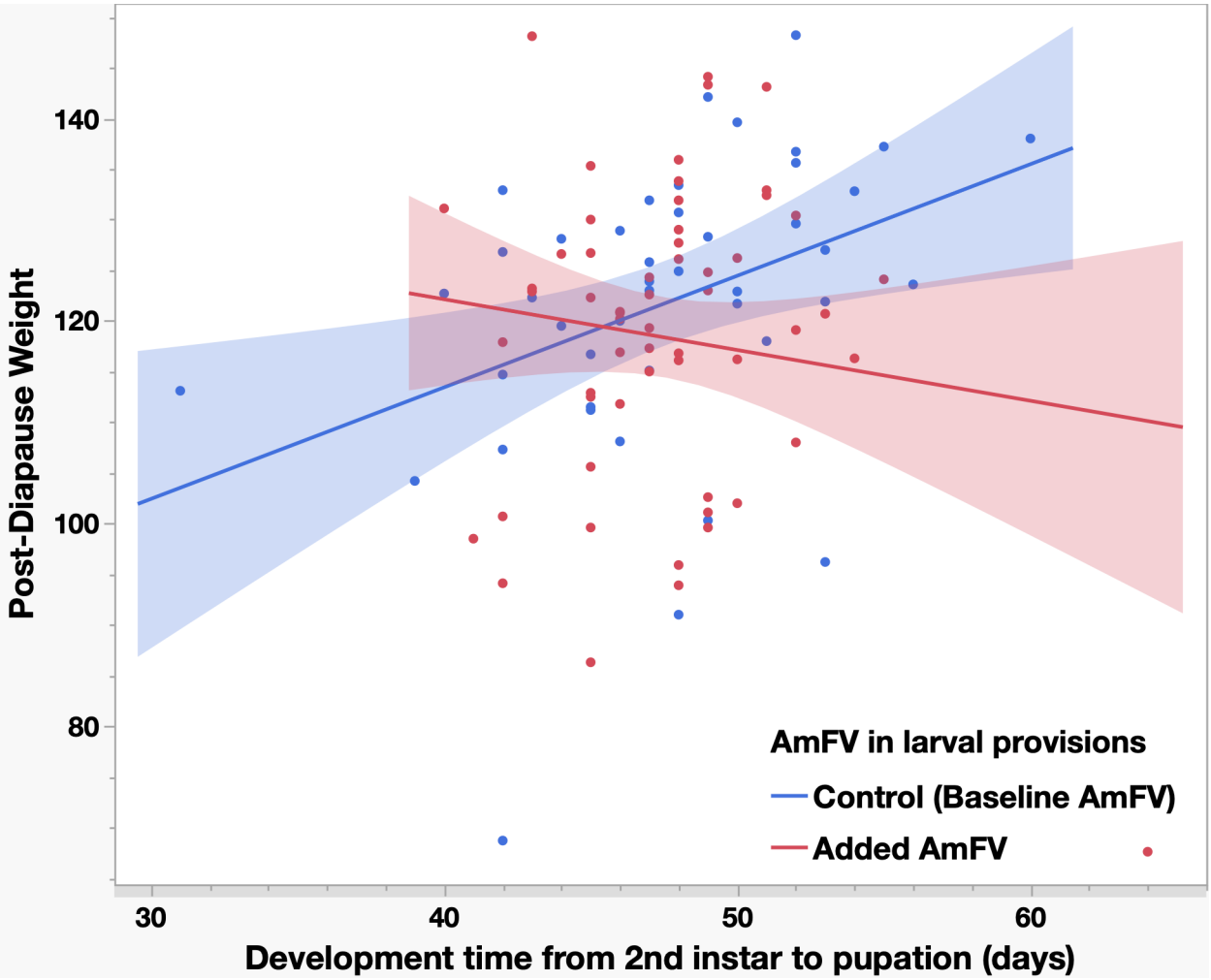


Figure 7, Williams, Schaeffer, and Cox-Foster



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OSSAmFVSupptables17.xlsx