1 **Long term effects of carbaryl exposure on antiviral immune responses in *Xenopus laevis***

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16 **Running Title:** Carbaryl effects on *Xenopus* anti-ranaviral immunity

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18 **Keywords:** Water pollutants, ranavirus, antiviral immunity, immune toxicant

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ABSTRACT

Water pollutants associated with agriculture may contribute to the increased prevalence of infectious diseases caused by ranaviruses. We have established the amphibian *Xenopus laevis* and the ranavirus Frog Virus 3 (FV3) as a reliable experimental platform for evaluating the effects of common waterborne pollutants, such as the insecticide carbaryl. Following 3 weeks of exposure to 10 ppb carbaryl, *X. laevis* tadpoles exhibited a marked increase in mortality and accelerated development. Exposure at lower concentrations (0.1 and 1.0 ppb) was not toxic, but it impaired tadpole innate antiviral immune responses, as evidenced by significantly decreased TNF- α , IL-1 β , IFN-I, and IFN-III gene expression. The defect in IFN-I and IL-1 β gene expression levels persisted after metamorphosis in froglets, whereas only IFN-I gene expression in response to FV3 was attenuated when carbaryl exposure was performed at the adult stage. These findings suggest that the agriculture-associated carbaryl exposure at low but ecologically-relevant concentrations has the potential to induce long term alterations in host-pathogen interactions and antiviral immunity.

1. Introduction

Aquatic vertebrates are increasingly exposed to a large number of water pollutants associated with agriculture [1-3]. These pollutants are detectable in most aquatic habitats, but their long-term effects at environmentally relevant levels on immune function remains unclear. Aquatic vertebrates are also exposed to circulating viruses and other pathogens, and there is some evidence suggesting associations between exposures to pollutants and greater susceptibility to circulating pathogens [4-6]. We have established the amphibian *Xenopus laevis* and the ranavirus Frog Virus 3 (FV3) as a reliable experimental platform for evaluating the effects of common waterborne pollutants on antiviral immune defenses across the lifespan.

Ranaviruses are contributing to the worldwide amphibian decline. Notably, ranavirus infections have not only become more prevalent over the past decade, but have also markedly increased in host range in amphibians and other ectothermic vertebrates. Indeed, besides more than 100 species of amphibians, there are a growing number of reptile and fish species reported to be infected by ranavirus pathogens (Chinchar et al., 2009; see eBook [7]). While emerging infectious diseases caused by ranavirus pathogens are concerning for biodiversity and aquaculture, they also raise questions about efficacies of antiviral immunity in amphibians in particular and aquatic vertebrates in general. Given the deleterious effects of environmental pollutants on animal development and physiology, it is important to evaluate more thoroughly their possible impact on immune function, and thus susceptibility to viral pathogens.

Pesticides have been proposed to represent a major cause of amphibian declines [8-11]. However, to date experimental evidence of the role played by environmental pollutants remains scant. In part this is due to the complexity of interactions between contaminants, pathogens and hosts. Some field studies have established correlations between the amounts of pesticides and the

decline of frog species [12]. Other studies have reported cumulative and interactive toxic effects of pesticides at low concentrations on amphibian development and survival [13-15]. Concerning the immune function, we have previously shown that water containing the herbicide atrazine at subtoxic level induces long lasting antiviral immune deficits in *X. laevis* [16]. The interpretation was that exposure even at low levels during early life can result in persistent effects that negatively impact immune defenses to pathogens such as FV3. To further address this possibility, we have examined the effects of another prominent water contaminant, the insecticide carbaryl.

Carbaryl (1-Naphthyl-N-methylcarbamate) is a pesticide used worldwide, and it has a tendency to reach water sources via agricultural runoffs. The U.S. Environmental Protection Agency (EPA) considers a maximum contaminant level for carbaryl at 40 parts-per-billion (ppb, 40 µg/L). Notably, the yearly average of carbaryl levels found in the Rochester NY area, is 0.3 ppb ([http://www.ewg.org/tap-water/whatsinyourwater/2021/NY/NewYork/ Carbaryl/](http://www.ewg.org/tap-water/whatsinyourwater/2021/NY/NewYork/Carbaryl/)) but can be as high as 1 ppb, and in regions with intensive pesticide drifts (e.g., Colorado), levels up to 16.5 ppb have been reported (cdpr.ca.gov/docs/emon/pubs/fatememo/carbaryl.pdf). Exposure to carbaryl has been linked to mutagenesis, disruption of hormone function (endocrine disruptor), and alteration in the immune system of humans (npic.orst.edu/factsheets/carbgen.pdf). Carbaryl can also be toxic to a variety of non-targeted species (birds, fish and amphibians). In anuran amphibians, the minimum lethal concentration is estimated to be 4.8 mg/L (4.8 ppm) [17]. However, aquatic animals such as amphibians are continuously exposed to water pollutants and therefore, may endure adverse health effects of carbaryl even at lower doses, which may become more critical during infectious diseases. Indeed, carbaryl appears to be more toxic for fish (rainbow trout) with an LC₅₀ of 1.4 mg/L (1.4 ppm) for a 96 hr exposure time. Exposure to a

sublethal dose of carbaryl has been shown to reduce host skin peptide defenses of several anurans species, which may increase susceptibility to infection by skin pathogens such as the chytrid fungus (*Batrachochytrium dendrobatidis*) [17-19]. To examine in more detail the potential immunomodulatory effects of carbaryl, we took advantage of the *X. laevis*/FV3 model system [20-22]. Specifically, we tested the hypothesis that developmental exposure at the tadpole stage to levels of carbaryl in the water that are at or below current measured levels in the environment results in alterations of antiviral immunity later in life, thus increasing susceptibility to pathogens such as FV3.

2. Materials and Methods

2.1. Animals

All outbred *Xenopus laevis* tadpoles and adult frogs were acquired from the *X. laevis* research resource for immunology at the University of Rochester (<http://www.urmc.rochester.edu/mbi/resources/Xenopus/>). For tadpole survival and gene expression experiments, stage 50 and 56 tadpoles were used, respectively [23]. One-year-old frogs were used for all adult experiments. All animals were handled in accordance with stringent laboratory and University Committee on Animal Research regulations (Approval number 100577/2003-151).

2.2. Carbaryl exposure

Highly purified carbaryl (Chem Service, West Chester, PA) was dissolved in DMSO as an initial stock solution from which subsequent working solutions were prepared. Two-week old (developmental stage 50; [24]) tadpoles (10 to 25 individuals per group depending of the experiment) were exposed for 3 weeks to 0.1, 1.0 or 10.0 ppb carbaryl in 4L containers (Fig. 1A). A concentration of 0.5% DMSO equivalent to the highest dose of carbaryl was used as a control in all experiments. The water and fresh carbaryl (or DMSO for control) were changed every week for each treatment group. All tadpoles were then transferred into 4L containers of clean water for recovery during one week to minimize possible stress due to the treatment. Survival following exposure to carbaryl was determined on groups of 25 individuals, whereas effects on developmental stages were monitored every day until metamorphosis completion for groups of 10 individuals. For adult treatment, one-year old outbred adults were exposed to 0.1, 1.0 or 10.0 ppb carbaryl in 2 L of water for 3 weeks.

2.3. Frog Virus 3 stocks and infection

Fathead minnow cells (FHM; American Type Culture Collection, ATCC No.CCL-42) and baby hamster kidney cells (BHK-21, ATCC No. CCL-10) were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), streptomycin (100µg/mL), and penicillin (100 U/mL) with 5% CO₂ at 30°C and 37°C, respectively. FV3 was grown using a single passage through FHM cells or BHK-21 cells and was subsequently purified by ultracentrifugation on a 30% sucrose cushion. Plaque assays on a FMH or BHK-21 cell monolayers were used to quantify FV3. Tadpoles were infected by intraperitoneal (i.p.) injection with 1x10⁴ plaque forming units (PFU) of FV3 in 10µL aliquots of amphibian phosphate buffered saline (APBS). Post-metamorphic one year-old frogs were infected by i.p. injection of 1x10⁶ PFU in 100 µL. For

all i.p. infections, uninfected control animals were mock-infected (i.p.) with an equivalent volume of APBS. One to 6 days post-infection (dpi), animals were euthanized using buffered MS-222 for kidney extraction (Fig. 1, bottom panel).

2.5. Quantitative gene expression analyses

RNA and DNA were extracted from frog kidneys using Trizol reagent, following the manufacturer's protocol (Invitrogen). Total RNA (0.5 µg of in 20 µl) was used to synthesize cDNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and 1 µl of cDNA template was used in all RT-PCRs and 250 ng DNA for PCR. Minus RT controls were included for every reaction. A water-only control was included in each reaction. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. Sizes of the products were determined using standardized markers of 1kb plus from Invitrogen (Carlsbad, CA).

The qPCR analysis was performed using the ABI 7300 real-time PCR system with PerfeCT SYBR Green FastMix, ROX (Quanta) and ABI sequence detection system software (SDS). GAPDH endogenous control was used in conjunction with the $\Delta\Delta CT$ method to analyze cDNA for gene expression. FV3 viral loads were assessed by absolute qPCR by analysis of isolated DNA in comparison to a serially diluted standard curve. Briefly, an FV3 DNA Pol II PCR fragment was cloned into the pGEM-T vector (Promega). This construct was amplified in bacteria, quantified and serially diluted to yield 10^{10} - 10^1 plasmid copies of the vDNA POL II. These dilutions were employed as a standard curve in subsequent absolute qRT-PCR experiments to derive the viral genome transcript copy numbers, relative to this standard curve. All primer sequences are listed in Table 1.

2.7. Statistical analysis

The Mann-Whitney *U* and ANOVA test were used for statistical analysis of expression and viral load data. Analyses were performed using a Vassar Stat online resource (<http://vassarstats.net/utest.html>). Statistical analysis of survival data was performed using a Log-Rank Test (GraphPad Prism 6). A probability value of $p < 0.05$ was used in all analyses to indicate significance.

3. Results

3.1. Effects of carbaryl exposure on *X. laevis* development and survival

We first determined whether developmental exposure to sublethal dose of carbaryl had any effect on survival and development in *X. laevis*. We exposed two-week old tadpoles (developmental stage 50; [23]) to 3 different concentrations of carbaryl (10, 1.0 and 0.1 $\mu\text{g/L}$) for 3 weeks, then after transfer to clean water we monitored the survival, changes in morphology, and time to complete metamorphosis and reach adult stages (Fig 1A). Although carbaryl exposure did not induce any significant mortality during or following exposure to 0.1 and 1.0 ppb, the higher concentration (10 ppb) did result in significant death in the weeks after the carbaryl treatment (Fig. 1B). No marked morphological alterations were noted. However, exposure to 10 ppb carbaryl did result in a significant acceleration of development, which led to a markedly shorter time for completion of metamorphosis (Fig 1C). The median time for metamorphosis completion of the 10 ppb treated group was 4.5 weeks compared to 14 weeks for the control group, whereas the median time for 0.1 ppb (10.5 weeks) and 1.0 ppb (11 weeks) treated group was slightly but not significantly shorter.

3.2. Effects of carbaryl exposure on *X. laevis* tadpole susceptibility to FV3 infection

To assess whether carbaryl exposure alters tadpole susceptibility to FV3 infection, two-week old tadpoles that were exposed for 3 weeks to the contaminant were infected with FV3 by i.p. injection and then viral loads in the kidneys (main site of FV3 replication in *X. laevis*) were determined by absolute qPCR of FV3 DNA Pol II on total DNA at 6 dpi, which correspond to the peak of FV3 infection (Fig. 2A). There was no statistically significant difference in the viral loads among treatment groups. Viral loads in other organs including spleen, intestine and peritoneal leukocytes (suppl. Fig. 1) were also comparable among treatment groups.

3.3. Effects of carbaryl exposure on *X. laevis* tadpole antiviral immunity

We next determined whether exposure to carbaryl altered the expression of three signature antiviral genes (TNF α , IL-1 β , and IFN-I) in pre-metamorphic tadpoles (stage 54-56) infected with FV3. Based on our prior study [21], we focused on kidney (main target organ of FV3 in *X. laevis*) at 3 dpi (early detectable innate antiviral immune response) and 6 dpi, which correspond to the peak of anti-FV3 immune response gene expression in the kidneys. Because of the marked decrease in tadpole survival following exposure to 10 ppb of carbaryl, only tadpoles exposed to 0.1 and 1 ppb were used. Notably, using qRT-PCR, expression of both IFN-I and IL-1 β genes was significantly (over a log) decreased in infected tadpoles at both 3 and 6 dpi and for both 0.1 and 1.0 ppb of carbaryl, as compared to DMSO controls (Fig. 3). It is noteworthy that alteration of IFN-I gene expression was not only detected in infected animals but also at steady state in uninfected animals. For TNF α , presumably because of the slower kinetics of gene expression

response to infection, a statistically significant decrease in transcript levels was only detected at 6 dpi for 0.1 ppb.

Since type III IFN or IFN- λ (IFN-III) has been shown to be involved during early anti-FV3 responses in tadpoles [25], we monitored changes in its expression during FV3 infection in the different tadpole exposure groups. Exposure to both 0.1 and 1.0 ppb carbaryl resulted in a significant decrease in IFN-III gene expression at 3 but not at 6 dpi. In contrast, for the transcript level of IFN-III at steady state in animals exposed to carbaryl there was trend to be higher that was however not statistically significant compared to control. We also assessed the expression response of the anti-inflammatory gene IL-10 and found a significant decrease at 3 dpi for animals exposed at both 0.1 and 1.0 ppb of carbaryl. We conclude that exposure to carbaryl for a limited time and at relatively low (sublethal) concentration markedly alters the kinetics and amplitude of antiviral gene expression response in tadpoles.

3.4. Effects of carbaryl exposure on *X. laevis* adults

Given the effects of carbaryl exposure on tadpole antiviral immunity, we next determined whether exposure to the same concentration would have a similar effect on antiviral immunity in mature adult frogs. Accordingly, 1 year-old frogs were exposed to 0.1 1.0 or 10.0 ppb carbaryl in their water for 3 weeks, and then infected with FV3. No adult frogs died from FV3 infections throughout the course of these experiments and no marked changes in animal morphology or behavior was observed (data not shown). Similarly, viral replication was not significantly altered by any treatment as shown by comparable viral loads at 3 dpi (Fig. 2B).

Since, innate immune responses to FV3 occur faster in *X. laevis* adults compared to tadpoles [26], we assessed gene expression response at 1 dpi. Interestingly, although carbaryl exposure at

all 3 concentrations did not significantly alter TNF- α and IL-1 β gene expression response to FV3, it impaired the FV3-induced IFN-I gene expression response at this point in time (Fig. 4). No significant alterations in gene expression for IFN-III and IL-10 were detected either (suppl. Fig. 2).

3.5. *Effects of developmental exposure to carbaryl on X. laevis after metamorphosis*

To determine whether alterations in antiviral immune responses induced by carbaryl were long-lasting, we examined treated tadpoles after they completed their metamorphosis. Specifically, two-week old pre-metamorphic tadpoles (Stage 50) were exposed to carbaryl for 3 weeks, then transferred in clean water and reared for 12 to 15 weeks to post-metamorphic froglets (Fig. 1A). Subsequently, frogs were infected (ip) with FV3 to assess their susceptibility and immune responses. Interestingly, while there was no effect on viral load in carbaryl-exposed tadpoles infected at tadpole stages one week after the treatment, there was a modest but statistically increased viral replication in kidneys of carbaryl-exposed tadpoles when they were infected at a later time after metamorphosis completion (Fig. 2C). Furthermore, this increased susceptibility was detected at the 3 doses of carbaryl exposure. In addition, long lasting alteration induced by carbaryl exposure was detected for the IFN-I expression response, which was significantly impaired from 1 to 6 dpi in the group treated with 0.1 ppb carbaryl and at 6 dpi for the 1.0 ppb treated group (Fig. 5). The expression response of IL-1 β was also decreased at 1 dpi for both 0.1 and 1.0 treated groups, whereas there were no significant changes in the TNF α gene expression profiles across the treatment groups. No significant alteration was found for the expression of IFN-III and IL-10 at steady state or after FV3 infection (suppl. Fig. 2). These data suggest that exposure to low sublethal levels of carbaryl during larval development induces long-lasting,

possibly developmentally related, alteration of antiviral innate immune response in young post-metamorphic adult frogs.

4. Discussion

Results from this study suggest that the potential immunotoxicity of certain water contaminants such as the widely used insecticide carbaryl may be more complex than usually considered. Notably, our data indicate that even at a concentration as low as 0.1 µg/L (0.1 ppb), which is 48,000x below the minimum lethal concentration for amphibians, developmental exposure to carbaryl induces alterations in antiviral immune gene expression responses that persist for several months through metamorphosis. Expression responses of the antiviral IFN-I gene as well as the pro-inflammatory genes IL-1β, and to a lesser level TNFα, were decreased upon FV3 infection both in tadpoles and in adults that were exposed to carbaryl at tadpole stages. Interestingly, viral load in kidneys was also significantly increased in adults developmentally exposed to carbaryl, but not in tadpoles nor in adults that were exposed at the adult stage. This suggests that carbaryl may affect the developmental process leading to the mature adult innate immune system after metamorphosis. While there has been some investigation of T and B cell remodeling during metamorphosis (reviewed in [27], little is known about changes undergone by innate immune cells. Nevertheless, tadpole and adult macrophages are phenotypically (e.g., absence of class I expression in tadpole macrophages) and functionally (low IL-34 expression by tadpole macrophages) distinct, suggesting that some differentiation occurs during metamorphosis.

4.1. Multiple possible mechanisms for immunotoxicity of carbaryl

Carbaryl disrupts the nervous system by blocking acetylcholinesterase. Specifically, it adds a carbamyl to the active site of the enzyme, blocking acetylcholine from properly getting into the active site and getting cleaved. However, effects of cholinesterase inhibitors have been reported at doses below those that are neurotoxic, suggesting they may act via other mechanisms too [28]. Thus, it is possible that the effects of carbaryl on antiviral immune defenses are not mediated by cholinesterase inhibition but rather via disruption of other less well characterized signaling pathways. For example, although the molecular targets remain to be fully defined, carbaryl alters signaling down stream of pattern recognition molecules and NF- κ B [29, 30]. Carbaryl has also been shown to act as an endocrine disruptor in mammalian and non-mammalian species, suggesting integrated processes that could be disrupted and lead to aberrant immune responsiveness [31-33]. The differential effects of carbaryl exposure on tadpoles versus post-metamorphic frogs, combined with emerging evidence that it also acts via non-acetylcholinesterase mediated mechanisms, suggests that these effects on anti-viral immune defenses may arise by more than one mechanism, and the precise mechanism may depend on the developmental stage at the time of exposure. In addition, it is possible that metabolites of carbaryl such as α -naphthol are causing some of the effects on the developing or mature immune system.

4.2. Persistent alterations of immune function induced by developmental exposure to carbaryl

The potential developmental modulation of leukocyte lineages by carbaryl in *X. laevis* is consistent with its reported endocrine disrupting activity [34]. Amphibian metamorphic development is controlled by the thyroid hormone (reviewed in [35]), although little is known

about hormonal control of the immune system transition besides MHC class I and II genes (reviewed in [36]). The possibility that carbaryl can affect leukocyte development is also consistent with the observed effect of carbaryl at a higher (10 µg/L or 10 ppb) dose, which considerably shortens the time to metamorphic completion. Similar acceleration of development induced by carbaryl has been reported in *Bufo bufo* [37]. It will be interesting to examine in more detail whether developmental exposure to carbaryl affect myeloid lineage subsets in adult frogs.

4.3. Acute effects of carbaryl on antiviral immunity

In addition to its developmental effects, our data suggest that carbaryl may also have a direct acute impact on antiviral IFN responses in tadpoles and adults, since exposure to two different doses of carbaryl showed a diminished expression response of the IFN-I gene during FV3 infection. Antiviral properties of the type I interferon response are well characterized in mammals [38, 39] and *Xenopus* [40]. In tadpoles, the IFN-I response to FV3 is more modest and delayed as compared to adult frogs, whereas tadpoles exhibit a rapid IFN-III response [25]. Thus, carbaryl-induced defects of both IFN-I and IFN-III responses may add up to an increase tadpole susceptibility to FV3 infection. In adult frogs, given a lesser involvement of IFN-III response, it is perhaps not so surprising that only IFN-I gene expression is affected by carbaryl. It is noteworthy that although IFN gene expression alteration did not have a marked effect on viral replication either in tadpoles or adult frogs, it remains possible that the lower IFN-I (and IFN-III in tadpoles) response results in more extensive tissue damages and/or delay in viral clearance. For example, lower and delayed IFN-I gene expression response in transgenic *X. laevis* line deficient of innate T cells correlates inefficient viral clearance with more damage in kidneys

[41]. Thus, it will be relevant to assess more thoroughly the consequence of poor IFN-I gene expression response induced by carbaryl exposure on pathogenesis and host fitness.

Acknowledgements

We thank Tina Martin for animal husbandry and Dr. Eva-Stina Edholm for her critical review of this manuscript. This work was supported by an R24-AI-059830 grant from the National Institute of Allergy and Infectious Diseases (NIH/NIAID R24-AI-059830 from NIH, a IOS-1456213 grant from the National Science Foundation and a D14ZO-084 grant from the Morris Foundation, and the Rochester Environmental Health Science Center (NIH/NIEHS P30-ES01247) and Toxicology Program (T32-ES07026).

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FIGURE LEGENDS

FIG 1. (A) Schematic of carbaryl treatment strategy. (B) Survival curve of tadpoles determined for groups of 25 tadpoles after 3 weeks exposure to 0, 0.1, 1.0 or 10 ppb carbaryl. Note that no significant death was recorded during the 3 weeks exposure to carbaryl at any of doses tested. (C) Fraction in percent of animals (10 individuals per treatment group) reaching metamorphic completion (stage 66) over time following carbaryl exposure. (#) $P < 0.05$ significant differences relative to DMSO treated only controls using one-way ANOVA test and Log-Rank Test (GraphPad Prism 6).

FIG 2. FV3 genome copy number at 3 dpi determined by absolute qRT-PCR in kidneys of (A) tadpoles and (B) adults exposed to 0, 0.1, 1.0 or 10 ppb carbaryl. (C) Kidneys of post-metamorphic froglets that were exposed to carbaryl at tadpole stage. Horizontal bars denote average $\pm$ SEM. There was no statistical difference between treatment groups in A and B, but statistical difference was noted in the adults exposed to carbaryl as tadpoles compared to DMSO controls as shown in panel C; $P < 0.05$ using one-way ANOVA and Mann-Whitney U Test.

FIG 3. Change in expression by qRT-PCR of pro-inflammatory (TNF- α and IL-1 β), anti-inflammatory (IL-10) and antiviral (IFN-I and IFN-III) genes in kidneys of tadpoles exposed to 0.1 or 1.0 ppb carbaryl at 0, 3 or 6 days post-FV3 infection. Results are average $\pm$ SEM comprised of 6 individuals per group and are representative from two different experiments. Statistical significance (one-way ANOVA and Mann-Whitney U Test): (#) $P < 0.05$ between

uninfected and infected DMSO control group; (*) $P < 0.05$, ** $P < 0.001$ between respective uninfected and infected groups. Gene expression is represented as fold increase (RQ: relative quantification) relative to GAPDH endogenous control and standardized to uninfected DMSO controls (fixed as 1).

FIG 4. Change in expression by qRT-PCR of pro-inflammatory (TNF- α and IL-1 β) and antiviral (IFN-I) genes in kidneys of one-year old *X. laevis* adult exposed to 0.1, 1.0 or 10 ppb carbaryl at 0 or 1 days post-FV3 infection. Results are average $\pm$ SEM comprised of 3 individuals. Statistical significance (one-way ANOVA and Mann-Whitney *U* Test): (#) $P < 0.05$ between uninfected and infected DMSO control group; (*) $P < 0.05$ between respective uninfected and infected groups. Gene expression is represented as fold increase (RQ: relative quantification) relative to GAPDH endogenous control and standardized to uninfected DMSO controls.

FIG 5. Change in expression by qRT-PCR of pro-inflammatory (TNF- α and IL-1 β) and antiviral (IFN-I) genes in kidneys of post-metamorphic *X. laevis* that were exposed at tadpole stage to 0, 0.1, 1.0 or 10 ppb carbaryl at 0, 1, 3 and 6 days post-FV3 infection. Results are means $\pm$ SEM comprised of 3 individuals. Statistical significance (one-way ANOVA and Mann-Whitney *U* Test): (#) $P < 0.05$ between uninfected and infected DMSO control group; (*) $P < 0.05$, ** $P < 0.001$ between respective uninfected and infected groups. Gene expression is represented as fold increase (RQ: relative quantification) relative to GAPDH endogenous control and standardized to uninfected DMSO controls.

Table 1. List of primer sequences

PRIMER	SEQUENCE (5'- 3')
vPOL II	F: ACGAGCCCGACGAAGACTACA R: TGGTGGTCCTCAGCATCC T
GAPDH	F: GACATCAAGGCCGCCATTAAGACT R: AGATGGAGGAGTGAGTGTCAACCAT
IFN-I	F: GCTGCTCCTGCTCAGTCTCA R: GAAAGCCTTCAGGATCTGTGTGT
IFN- III (λ)	F: TCCCTCCCAACAGCTCATG R: CCGACACACTGAGCGGAAA
IL -1β	F: CATTCCCATGGAGGGCTACA R: TGACTGCCACTGAGCAGCAT
IL-10	F: TGCTGGATCTTAAGCACACCCTGA R: TGTACAGGCCTTGTTACGCATCT
TNF-α	F: TGTCAGGCAGGAAAGAAGCA R: CAGCAGAGCAAAGAGGATGGT

F: Forward; R: Reverse

Fig. 2

Fig. 3

Fig. 4

Fig. 5