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## Autophagic chemicals effect to Atg8 and rice stripe virus relative expressions, and *Wolbachia* relative density in *Laodelphax striatellus* (Hemiptera: Delphacidae)

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**Abstract:** *Laodelphax striatellus* is one of the most important pest insects and vectors of rice stripe virus (RSV), which causes rice stripe disease (RSD) in rice plants. *L. striatellus* is infected by *Wolbachia*. RSV and *Wolbachia* may be regulated by a cell defense mechanism within insect cells called autophagy. We utilized autophagic chemicals to predict the relationships between Atg8 (an importance of autophagy-related genes), *Wolbachia*, and RSV within *L. striatellus*. The relative expressions and density level of Atg8, RSV, and *Wolbachia* were determined by three autophagy inducers and two inhibitors. We examined the effect of chemicals on the host. We report that *Wolbachia* relative densities were always higher than RSV relative expression. The presence of *Wolbachia* possibly confounds the linearity relationship between Atg8 and RSV in *L. striatellus*. Spermidine and 3-methyladenine were found to be better autophagic chemicals for reducing the RSV load and allowing a normal host's life. Still, they do not affect the *Wolbachia* density. This discovery demonstrates the potential to manipulate autophagy-related genes as an alternative type of RSD control.

**Key words:** Atg8, *Wolbachia*, rice stripe virus, small brown planthopper

### 1. Introduction

The small brown planthopper *Laodelphax striatellus* (Fallén) (Hemiptera: Delphacidae) is one of the most important rice pest insects in temperate and subtropical areas (Lijun et al., 2003). *L. striatellus* is infected by the rice stripe virus (RSV) (Lijun et al., 2003) and *Wolbachia* (Li et al., 2017). *L. striatellus* exposed to RSV by sucking fluid from infected host plants using a piercing-sucking mouthpart, leading to extensive viral transmission (Lijun et al., 2003). Rice stripe disease (RSD), a rice plant disease caused by RSV, induces severe or even crippling losses in many temperate rice cultivation areas (Lijun et al., 2003; Zhu et al., 2017). RSV, which belongs to the *Tenuivirus* genus and a negative strand RNA genome (1998; Sastry, 2014), is transmitted by *L. striatellus* in a circulative/persistent manner which propagates not only in host plants but also in host insects by systematically invading several tissues (Deng et al., 2013, Wu et al., 2014).

*Wolbachia* is a maternally inherited gram-negative bacteria (Serbus et al., 2008) and infects many arthropods and nematodes (Bandi et al., 2001). *Wolbachia* has been found in the cells of various tissues such as the head, thorax, and legs (Hui et al., 2010) and predominantly in the reproductive organs (Dobson et al., 1999). *Wolbachia*

manipulates host insects' reproduction in various ways, such as cytoplasmic incompatibility (CI) (Noda et al., 2001; Tagami et al., 2006), male-killing (Unckless and Jaenike, 2012), feminization (Narita et al., 2007; Le Clec'h et al., 2014), and thelytokous parthenogenesis (Arakaki et al., 2001; Zhu et al., 2007). Besides the host reproductive manipulation, *Wolbachia* affects the host in various ways. One of interests is limiting the replication of viruses (Mohanty et al., 2016; Tan et al., 2017). *Wolbachia* was reported to be an intermediate in the limiting and blocking of dengue (Moreira et al., 2009; Blagrove et al., 2011), zika (Dutra et al., 2016; Schultz et al., 2018), and chikungunya (Moreira et al., 2009; Tan et al., 2017) viruses within *Aedes aegypti* (Moreira et al., 2009; Tan et al., 2017) and *Aedes albopictus* (Blagrove et al., 2011). Mosquitoes infected by *Wolbachia* were released into the field to control wild-type vectors. This program has been carried out in Australia (Nguyen et al., 2015), Vietnam (Nguyen et al., 2015), and Indonesia (Reilly et al., 2019). All of the mentioned studies above regarding the relationship between *Wolbachia* and viruses related to human health, while relationships in the agricultural sector just have been started by Gong et al. (2020) who presented that *wStri* from *L. striatellus* inhibited the infection transmission of Rice ragged stunt

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virus after introduced to uninfected *Nilaparvata lugens*. This phenomenon encouraged us to conduct a study in this field. We focus on *Wolbachia* as a relevant symbiont controlling arbovirus in vector populations instead of CI as incompatible insect techniques that control the vector population.

Viruses and bacteria activate host cell autophagy (referred to as macroautophagy), a conserved cell defense mechanism and regulator of homeostasis (Voronin et al., 2012), which involves the degradation of damaged and unused organelles and proteins and defense against intracellular microorganisms (Levine and Mizushima, 2011). A previous study indicated that the activation of autophagy could reduce the bacterial load to the same extent as antibiotic therapy (Voronin et al., 2012) and regulate lipid metabolism to promote viral spreading and replication (Singh et al., 2009; Chen et al., 2017). Deehan et al. (2021) revealed that core autophagy proteins Atg1, Atg8, and a selective autophagy-specific protein Ref(2) p negatively regulate *Wolbachia* in the hub, a male gonad somatic cell type of *Drosophila melanogaster*. Due to the important role of autophagy in homeostatic cells, it was necessary to include the metabolic dynamic caused by the presence of *Wolbachia* and RSV. In this study, we investigated the relationships among Atg8 (an important of autophagy-related genes), *Wolbachia*, and RSV and the effect of three autophagy inducers and two inhibitors on *Wolbachia*, RSV, and *L. striatellus* as their host. Manipulation of autophagy related genes possibly reveals the autophagy as an alternative to RSD control.

## 2. Materials and methods

### 2.1. Insect rearing

*L. striatellus* infected with RSV and *Wolbachia* (wStri) strains were sourced from the National Agriculture and Food Research Organization, Japan. A population of around 50–100 individuals was reared in our laboratory as stock in cylinder clear cages (Ø 9.9 cm × h 16 cm) using healthy rice plants (*Oryza sativa*) as food under conditions of 16 h light and 8 h dark, relative humidity of 55%, and a temperature of 25 °C. When the rice plants were considered insufficient to provide the insects' nutrition demands, around 50–100 *L. striatellus* adults infected with RSV and *Wolbachia* were moved to a new cage with new healthy rice plants.

### 2.2. Treatment with autophagy inducers and inhibitors

Autophagy activity can be quantified by measuring the relative expression of Atg8 (Kabeya et al., 2004). To study the relationship between the relative expressions of Atg8 and RSV and the relative density of *Wolbachia*, we utilized metformin (MET; Lkt Laboratories, Inc., USA), rapamycin (RAP; Funakoshi, Japan), and spermidine (SPER; Cayman Chemical, USA) as autophagy inducers

(Poudel et al., 2017). As autophagy inhibitors, we applied 3-methyladenine (3MA; Santa Cruz Biotechnology, Inc., USA) and chloroquine (CQ; Fujifilm, Japan) (Poudel et al., 2017). Less than 24 h after emergence, we moved the 25–30 of adults to other cages (6 × 6 × 9.8 cm) for treatment. There was one cage per treatment. The host plants were sufficiently sprayed every day for three days, including the insects within the cage, with different concentrations of autophagy inducers and inhibitors. There were 2 mM and 8 mM of CQs (Poudel et al., 2017), 100 mM and 200 mM of METs (Tomic et al., 2011; Shi et al., 2012), 250 µM and 500 µM of 3MAs and RAPs (Bjedov et al., 2010), and 250 µM and 1 mM of SPERs (Voronin et al., 2012). The insects were collected after 3 days treatment, and the DNA and RNA of insects were isolated directly or stored at –80 °C before use.

### 2.3. DNA–RNA isolation and cDNA reverse transcription

The total DNA from pooled *L. striatellus* adults was extracted with the DNeasy Blood and Tissue Reagent Kit (Qiagen, Germany) based on the manufacturer's instruction. The DNA concentration was quantified using a Nanodrop 1000 (Thermo Scientific, USA). The 260/280 nm absorbance ratio was checked; 1.8 or above was considered acceptable (Jasbeer et al., 2009). Total RNA was extracted using the Nucleospin RNA isolation kit (Macherey-Nagel, Germany). Only the method of destroying insect tissue was modified according to the manufacturer's instructions. A power masher II machine (Nippi, Japan) was applied to crush around 15 mg of *L. striatellus* body tissue, which required around 10–15 individuals in the same ages of adults per DNA or RNA extraction. The 260/280 nm absorbance ratio of 2.0 for the RNA was considered to be good (Taylor et al., 2010). The RNA solution was stored at –80 °C or directly applied to cDNA reverse transcription.

*L. striatellus* cDNA was reverse transcribed from *L. striatellus* RNA by ReverTra Ace qPCR RT Master Mix random hexamers with the gDNA Remover master kit (Toyobo, Japan). Around 1.8 of cDNA ratio absorbed at a wavelength of 260/280 nm was used in this study (Jasbeer et al., 2009). The cDNA solution was stored at –20 °C or applied directly to qPCR.

### 2.4. Primers, PCR, and qPCR

The primers used for PCR were chosen based on the previous research (Folmer et al., 1994; Werren et al., 1995; Zhou et al., 1998; Yu et al., 2020), they were designed with NCBI GENBANK data using the BLAST primer search engine also. They were listed in Table.

*Wolbachia*, RSV, and Atg8 detection was conducted by GoTaq (Promega, USA). The reaction mixture was run on a Takara PCR Thermal Cycler Dice (Takara, Japan). The PCR cycling program, which was applied to all primers, involved denaturation at 98 °C (10 s), 35 cycles of annealing (98 °C (10 s), 55 °C (30 s), 72 °C (1 min)), and elongation at 72 °C (4 min).

**Table.** The primers used in this study.

Primers	Primer sequence (5' -3')	Annealing (°C)	Length (bp)	Reference
WSP	81F: tggccaataagtgatgaagaac 691R: aaaaattaacgctactcca	55	600	(Zhou et al., 1998)
wLstriq	F: ctccatacgttggtgttggtg R: cgagctccagcaagaggttt	55	157	This study
RSV	F: cacccttgagactgacac R: gtggtctgttctgctctgct	54	948	This study
qRSV	F: tcacccttgagactgaca R: tcttggtatccaaccgcc	55	173	This study
COI	LCO1490: ggtaacaatacataagatattgg HCO2198: taaacttcagggtgacaaaaaatca	52	710	(Folmer et al., 1994)
qCOI	F: cggtgccccagatatagcat R: cgtccggagtgaggagatgac	55	162	This study
Atg8	F: gaggaaaagtgaaggcgaga R: cgacggtgagatcagatggc	55	134	(Yu et al., 2020)

To obtain the qPCR primers, the sequencing fragments (the accession numbers are MK599162.1 for *Wolbachia*, EU931513.1 for RSV, and MK292977.1 for COI) were applied to the BLAST primer search engine. The constructed qPCR primers are listed in the Table. The qPCR Taqman II Reaction mixture was made with the TB Green Premix Ex Taq II (Tli RNaseH Plus) reagent kit (Takara Bio Inc., Japan). The reaction volumes are 12.5  $\mu$ L of TB Green Premix Ex Taq II, 1  $\mu$ L of forward and reverse primers, < 100 ng of DNA or cDNA templates, and 8.5  $\mu$ L sterile purified water (25  $\mu$ L of total reaction volume). It was run on a Thermal Cycler Dice Real-Time System II (Takara, Japan). The qPCR cycling program was 98 °C for 10 s, followed by 40 cycles of 98 °C for 10 s and 55 °C for 30 s (obtaining the Cq value). Assays were conducted as three technical replicates of qPCR reactions of the pooled sample for each target gene (Ge et al., 2020), and comparing the copy number of the *Wolbachia* gene to that of the *L. striatellus* cytochrome oxidase I (COI) gene (Watanabe et al., 2011).

### 2.5. Autophagic chemicals effect on host

Stage three after molting (less than 24 h) of *L. striatellus* nymphs was used in this study. Fifteen *L. striatellus* nymphs were established in cages (6  $\times$  6  $\times$  9.8 cm) per group containing healthy rice plants. The cages were sprayed every day by autophagy inducers and inhibitors, except for the control, which was sprayed with water (see subsection 2.2). The number of live and dead insects was recorded every day until all insects died. The replication research method was based on Federer (1967). Five replications were used in this study.

### 2.6. Statistical analysis

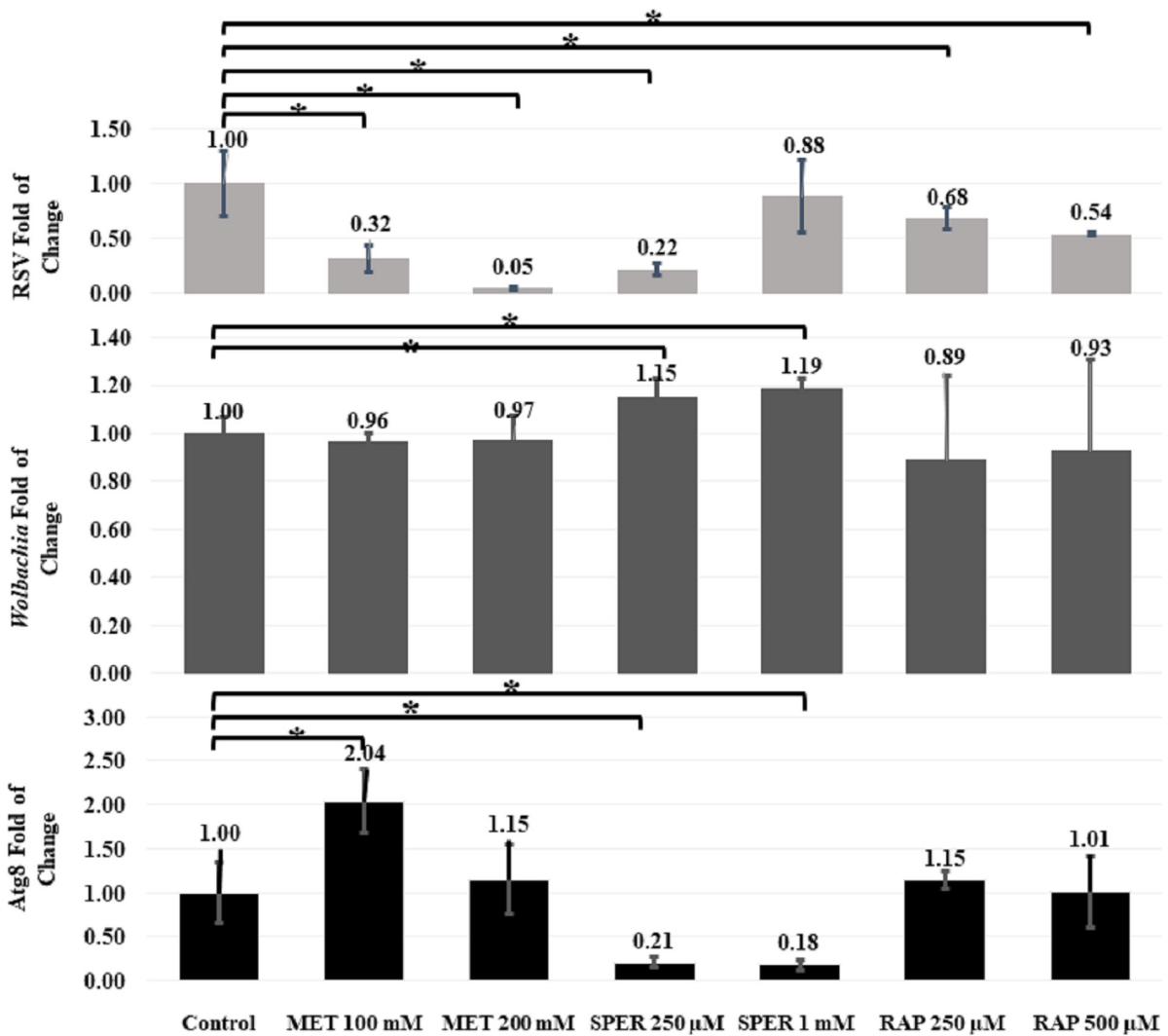
Statistical data analyses were performed using IBM SPSS statistics 16 software (IBM, USA). The significant differences between control and treatments were determined using the Student's t-test accompanied by Bonferroni correction. One-way analysis of variance (ANOVA) followed by the Tukey HSD comparison test was selected to determine the significant differences of autophagic chemicals' effect on insects life. The relative densities and expression levels of Atg8, *Wolbachia*, and RSV were analyzed by the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

### 3. Results

#### 3.1. Effect of autophagy inducers on Atg8 and RSV relative expressions and *Wolbachia* relative density level

The effects of autophagy inducers on the relative expressions and density levels of Atg8, *Wolbachia*, and RSV are shown in Figure 1. The relative expression of Atg8 after treatment with 100 mM of MET ( $\bar{x}$  = 2.04,  $t$  = 10.33;  $df$  = 4;  $p$  = 0.002) was significantly higher than that of the control. The relative expression of Atg8 after treatment with 250  $\mu$ M or 1 mM of SPER was significantly lower ( $\bar{x}$  = 0.21,  $t$  = -8.62;  $df$  = 4;  $p$  = 0.000 and  $\bar{x}$  = 0.18,  $t$  = -10.67;  $df$  = 4;  $p$  = 0.000) than that of the control. Other treatments (200 mM of MET ( $\bar{x}$  = 1.15,  $t$  = 1.78;  $df$  = 4;  $p$  = 1.000), 250 ( $\bar{x}$  = 1.15,  $t$  = 2.17;  $df$  = 4;  $p$  = 1.000) and 500  $\mu$ M of RAPS ( $\bar{x}$  = 1.01,  $t$  = 0.14;  $df$  = 4;  $p$  = 1.000)) showed that the relative expression of Atg8 did not differ significantly.

Treatment with 250  $\mu$ M ( $\bar{x}$  = 1.15,  $t$  = 3.65;  $df$  = 4;  $p$  = 0.018) or 1 mM ( $\bar{x}$  = 1.19,  $t$  = 4.27;  $df$  = 4;  $p$  = 0.029) of SPER increased the relative density of *Wolbachia*.



**Figure 1.** Comparison of Atg8, *Wolbachia*, and RSV relative expressions and densities. Vertical bar lines indicate the  $\pm$  of SD. Significant difference was determined by the Student's t-tests accompanied by Bonferroni correction (0.05). Control was used as a reference. Asterisks represent a significant difference in genes between treatment and control ( $*p < 0.05$ ).

Other treatments (100 and 200 mM of METs ( $\bar{x} = 0.96$ ,  $t = -0.85$ ;  $df = 4$ ;  $p = 1.000$  and  $\bar{x} = 0.97$ ,  $t = -0.32$ ;  $df = 4$ ;  $p = 1.000$ ); 250 and 500  $\mu\text{M}$  of RAPs ( $\bar{x} = 0.89$ ,  $t = -0.39$ ;  $df = 6$ ;  $p = 1.000$  and  $0.93$ ,  $t = -0.28$ ;  $df = 6$ ;  $p = 1.000$ ) were not associated with significant differences in the relative density of *Wolbachia* compared with the control (Figure 1).

Treatment with 100 or 200 mM MET reduced the RSV load ( $\bar{x} = 0.32$ ,  $t = -4.05$ ;  $df = 4$ ;  $p = 0.045$  and  $\bar{x} = 0.05$ ,  $t = -16.33$ ;  $df = 5$ ;  $p = 0.000$ ). Treatment with 250  $\mu\text{M}$  SPER also significantly lowered the relative density of RSV compared with the control ( $\bar{x} = 0.22$ ,  $t = -7.27$ ;  $df = 4$ ;  $p = 0.003$ ). A total of 250 and 500  $\mu\text{M}$  of RAPs lowered the relative density of RSV compared with the control also ( $\bar{x} = 0.68$ ,  $t = -0.62$ ;  $df = 5$ ;  $p = 0.000$  and  $\bar{x} = 0.54$ ,  $t = -1.50$ ;  $df = 5$ ;  $p = 0.000$ ). The effect of treatment with 1 mM SPER

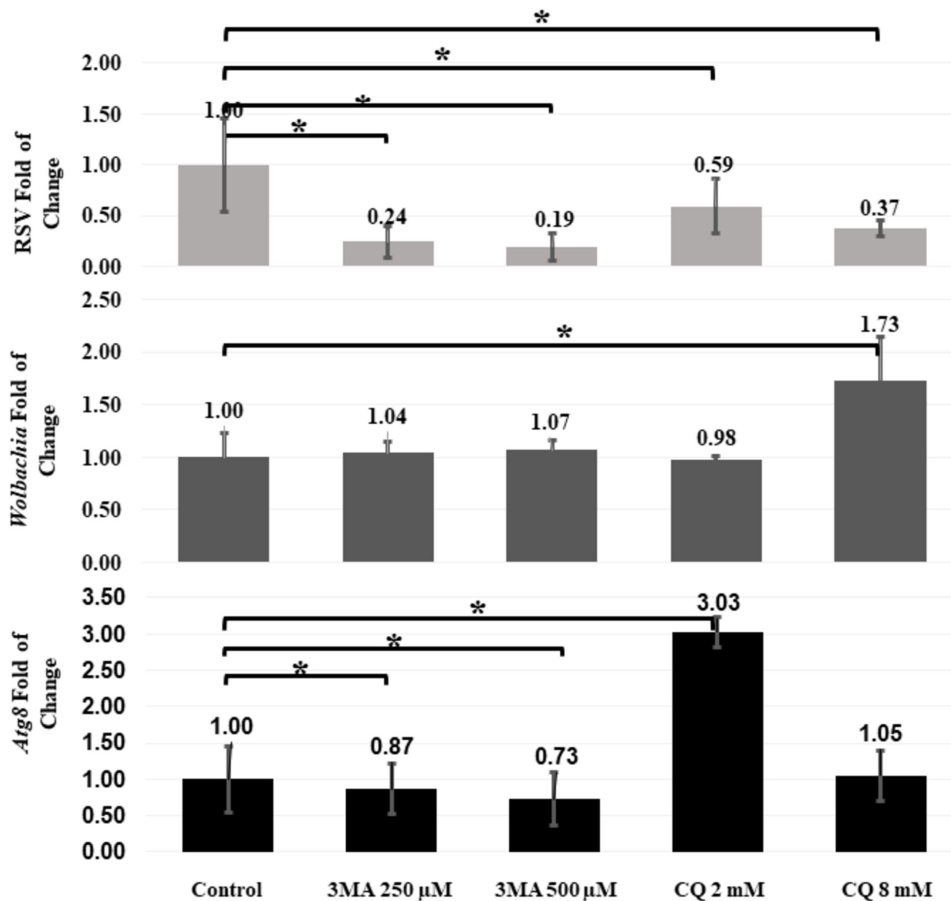
( $\bar{x} = 0.88$ ,  $t = 2.51$ ;  $df = 4$ ;  $p = 0.634$ ) was not significantly different (Figure 1).

### 3.2. Effect of autophagy inhibitors on Atg8 and RSV relative expressions and *Wolbachia* relative density level

Figure 2 shows that treatment with 250  $\mu\text{M}$  and 500  $\mu\text{M}$  3MAs ( $\bar{x} = 0.87$ ,  $t = -0.55$ ;  $df = 4$ ;  $p = 0.011$ ) and ( $\bar{x} = 0.73$ ,  $t = -16.17$ ;  $df = 4$ ;  $p = 0.039$ ) decreased the relative expression of Atg8 significantly, but there was no significant difference when was used. Treatment with 2 mM CQ increased the relative expression of Atg8 by about three-fold compared with the control ( $\bar{x} = 3.03$ ,  $t = 16.50$ ;  $df = 4$ ;  $p = 0.004$ ), but there was no significant difference with 8 mM CQ ( $\bar{x} = 1.05$ ,  $t = 0.21$ ;  $df = 4$ ;  $p = 1.000$ ).

The relative density of *Wolbachia* increased compared with the control following treatment with 8 mM CQ ( $\bar{x} =$





**Figure 2.** Comparison of Atg8, *Wolbachia*, and RSV relative expressions and densities. Vertical bar lines indicate the  $\pm$  of SD. Significant difference was determined by the Student's t-tests accompanied by Bonferroni correction (0.05). Control was used as a reference. Asterisks represent a significant difference in genes between treatment and control (\* $p < 0.05$ ).

1.73,  $t = 6.96$ ;  $df = 4$ ;  $p = 0.000$ ), and the other treatments (2 mM of CQ ( $\bar{x} = 0.98$ ,  $t = -0.58$ ;  $df = 4$ ;  $p = 1.000$ ), 250  $\mu\text{M}$  and 500  $\mu\text{M}$  of 3MAs ( $\bar{x} = 1.04$ ,  $t = 0.19$ ;  $df = 3$ ;  $P = 1.000$  and  $\bar{x} = 1.07$ ,  $t = -0.02$ ;  $df = 3$ ;  $p = 1.000$ ) were not associated with significantly different results (Figure 2).

According to Figure 2, all treatments decreased RSV. Treatment with 250  $\mu\text{M}$  or 500  $\mu\text{M}$  3MA ( $\bar{x} = 0.24$ ,  $t = -5.51$ ;  $df = 4$ ;  $p = 0.000$  or  $\bar{x} = 0.19$ ,  $t = -24.71$   $df = 4$ ;  $p = 0.000$ ) and 2 mM or 8 mM CQ ( $\bar{x} = 0.59$ ,  $t = -1.73$ ;  $df = 7$ ;  $p = 0.010$  and  $\bar{x} = 0.37$ ,  $t = -4.94$   $df = 4$ ;  $p = 0.007$ ) significantly decreased the relative expression of RSV compared with the control.

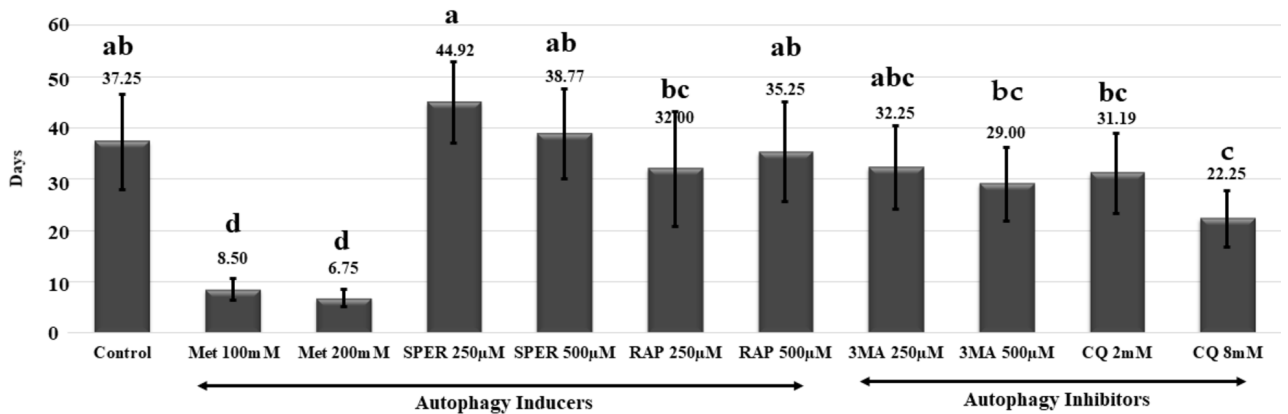
### 3.3. Autophagic chemicals effect on *L. striatellus*

Based on the analysis using one-way ANOVA ( $F = 15.96$ ;  $df = 43$ ;  $p = 0.000$ ), the autophagic chemicals' effect on *L. striatellus* differed depending on whether it was treated with autophagy inducers or inhibitors (Figure 3). Furthermore, the Tukey HSD comparison test indicated a decrease in insect life following treatment with 8 mM CQ, 100 mM, and 200 mM METs.

## 4. Discussion

Autophagy can be predicted by Atg8 expression (Kabeya et al., 2004; Schaaf et al., 2016), because of its presence in autophagic flux as Atg8 II, which is formed from Atg8 I (Kabeya et al., 2004). We assumed that the greater the Atg8 gene expression levels, the greater the amount of autophagic activity in the cells. In this study, we used autophagic chemicals to either induce or inhibit autophagy then the Atg8 expression was a proxy to estimate the chemical performance within *L. striatellus*. The treatments involved autophagy inducers (100 and 200 mM METs, 250  $\mu\text{M}$  and 1 mM SPERs, and 250 and 500  $\mu\text{M}$  RAPs) and inhibitors (250 and 500  $\mu\text{M}$  3MAs, and 2 and 8 mM CQs). The concentration of chemicals in this study was modified based on previous studies because of the different species used (Bjedov et al., 2010; Tomic et al., 2011; Shi et al., 2012; Voronin et al., 2012; Poudel et al., 2017).

Based on Figure 1, autophagy induction with 100 mM MET increased the relative expression of Atg8. SPERs decreased the relative expression of Atg8 and increased



**Figure 3.** Autophagic chemicals affect comparison on *L. striatellus* for three days. Each data point represents the average of four replications. Vertical bar lines indicate the  $\pm$  of SD. The significant differences among the treatments are indicated by different letters (one-way ANOVA  $p < 0.01$  and Tukey's comparison test).

the relative density of *Wolbachia* compared with the control. We suspect the SPERs acts more as a quorum sensing inducer of bacteria (Nasrallah et al., 2011) than an autophagy inducer, causing the increasing *Wolbachia* density, which tends to be accompanied by lower autophagy (Voronin et al., 2012; Kamalakannan et al., 2015). Further study in this area utilizing other insects and/or organisms is needed. Autophagy inhibition (Figure 2) with 500  $\mu$ M 3MA significantly decreased the concentration of Atg8, and treatment with 2 mM CQ increased Atg8 significantly. Although CQ was previously used as an autophagy inhibitor (Mauthe et al., 2018), our data showed that it actually induced autophagy. We suspect that the high expression of Atg8 was derived from the autophagic flux obtained in the preautolysosome stage (Kaushal, 2012). CQ only decreases the autophagic flux during autophagosome-lysosome fusion (Kaushal, 2012). CQ was previously identified as an autophagy inducer by Solomon and Lee (2009) in ovarian and chronic myelogenous leukemia cells. Geng et al. (2010) also confirmed that CQ induces autophagic vacuoles in glioblastoma.

SPERs and CQ 8mM, affected the density of *Wolbachia*, whereas (MET, RAP, 3MA, and CQ 2mM) had no effect (Figures 1 and 2). SPERs and 8mM CQ regulated the relative density of *Wolbachia*, which were associated with a significant increase in density compared with each control (Figures 1 and 2). There was no significant effect on METs, RAPs, 3MAs, and CQ 2mM. It is presumed that autophagy better tolerates the mutualistic situation at *Wolbachia* using *L. striatellus* as a native host. Clec'h et al. (2012) found that acute autophagic responses were not exhibited in native *Wolbachia* host species, whereas this phenomenon was observed in recipient host species. The study was conducted on the wVulC *Wolbachia* strain, transfected from terrestrial isopods as a native host (*Armadillidium vulgare*) to another phylogenetically

similar species (*Porcellio d. dilatatus*). Another possibility is the discrepancy in the dose of chemicals used in this study. Further research on the manipulation of autophagic chemical doses is needed.

SPERs add to the list of autophagy inducers capable of regulating *Wolbachia* which have previously been used RAPs to regulate and determine the size of the *Wolbachia* population (Voronin et al., 2012; Kamalakannan et al., 2015). Kamalakannan et al. (2015) reported that the induction of autophagy by RAPs mediated the degradation of Toll-Like Receptor-4 and protected monocytes from recombinant *Wolbachia* heat shock protein 60, which mediated the multiplication of *Wolbachia*. Interestingly, we found bottom-up and top-down mechanisms in the relationship between Atg8-*Wolbachia* relative expression and density by SPER treatment (Figures 1). When the Atg8 expression is low, the *Wolbachia* density is high and vice versa.

The relationship between autophagy and RSV has been studied previously. Yu et al. (2020) revealed that Atg8 and RSV run linearly. Whenever Atg8 expression is knocked down, RSV expression is also decreased. However, our results show that the linearity between Atg8 and RSV relative expressions is inconsistent. An increase in RSV did not accompany the increase of Atg8 with MET 100 mM and CQ 2 mM. It is suspected that the presence of *Wolbachia* confounds the linearity relationship between Atg8 and RSV, considering that *Wolbachia* relative densities are almost always higher than RSV relative expressions.

The relative density of *Wolbachia* was never lower than control, whereas the relative density of RSV was never higher than control except with 2 mM CQ (Figures 1 and 2). Indirectly, the fact suggests that *Wolbachia* possibly limits RSV development (Mohanty et al., 2016; Tan et al., 2017). Our results also support a previous report showed that *Wolbachia* from *L. striatellus* can limit the rice rugged

ragged stunt virus (RRSV) development after being introduced to *Nilaparvata lugens* as a vector of the virus (Gong et al., 2020).

Answering about RSV density decreased in almost all treatments, whether autophagy increased or *Wolbachia* decreased remains a mystery. So, further research is needed to clarify the relationship between *Wolbachia* and RSV. Regulating *Wolbachia*, but not autophagy, and seeing its effect on RSV must be an option. The suppression of *Wolbachia* or RSV within *L. striatellus* will be necessary to clarify the direct relationship between autophagy-*Wolbachia* and autophagy-RSV. However, it is difficult to remove autophagy, even though it can be done via RNAi or CRISPR techniques. It always exists in living cells as an intracellular innate immune system (Amano et al., 2006).

Regarding the relationship between *Wolbachia* and viruses, Teixeira et al. (2008) revealed that the presence of *Wolbachia* in *Drosophila melanogaster* decreased the burden of Nora and Flock virus' as RNA viral infections. This finding was also corroborated by Mohanty et al. (2016), who reported that *Wolbachia* induced resistance to diverse RNA viruses and protected flies from virus-induced mortality. This result provides a clue about the relationship between *Wolbachia* and RSV because RSV is an RNA virus also (Lijun et al., 2003). However, a confirmation study is needed, and this should include symbiont effects on RSV because *L. striatellus* is infected not only by *Wolbachia* but also by *Spiroplasma*, *Cardinium*, *Arsenophonus*, *Acinetobacter*, *Chryseobacterium*, *Serratia*, *Arthrobacter*, *Asaia*, and yeast-like endosymbionts (Li et al., 2017; Li et al., 2018; Zhang et al., 2020). Autophagy inducers and inhibitors may affect their relative densities, and expression levels may be affected by autophagy inducers and inhibitors. They may affect the relative density of *Wolbachia* and the relative density of RSV either directly or indirectly.

The goal of utilizing autophagy chemicals as alternative sources of RSD control must be achieved without adverse effects on *L. striatellus*. It must generally live as control and contribute to a strong reductive effect on the RSV burden. Based on the autophagic chemicals observation

data (Figure 3), treatment with 100 or 200 mM of MET or 8 mM of CQ decreases the insect's life compared with the control. We assume that the disparity in the life of *L. striatellus* may interrupt the life cycle, fecundity, and the pattern of oviposition (Zhang et al., 2015), and it would be interesting to do further study in this field. The other autophagy chemical treatments were not associated with any significant differences compared with the control. Treatment with 250  $\mu$ M of the autophagy inducer SPER and 500  $\mu$ M of the autophagy inhibitor 3MA reduced the relative density of RSV by up to 78% ( $\bar{x} = 0.22$ ,  $t = -7.27$ ;  $df = 4$ ;  $p = 0.000$ ) and 81% ( $\bar{x} = 0.19$ ,  $t = -24.71$   $df = 4$ ;  $p = 0.000$ ), and there was no significant difference in insect's life compared with the control ( $p = 0.999$  and  $p = 0.998$ ). Other chemicals, such as MET and CQ, were associated with a strong reduction in the RSV load in *L. striatellus*. Unfortunately, it was difficult to reach a definitive conclusion due to the lack of insect life data (1 mM replaced by 500  $\mu$ M of SPER) or the disparity in the insect's life caused by autophagic chemical treatments. In addition, a study on the cytoplasmic incompatibility (CI) level affected by autophagy is also important and interesting, because a decrease in the insect pest population is the goal of insect pest control.

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#### Contribution of authors

AG and YT conceived the study. AG performed most of the experiment. AKH reared insect samples and genetic research experiments. AG and YT wrote the paper. All authors edited the final manuscript.

#### Conflicts of interest

The authors declare no conflict of interest in this study.

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