Autophagic chemicals effect for male-killing Wolbachia, Atg8 and TOR genes in Ostrinia scapulalis (Lepidoptera:Crambidae)

Autophagic chemicals effect for Male-Killing *Wolbachia*, *Atg8* and *TOR* genes in *Ostrinia scapulalis* (Lepidoptera: Crambidae)

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Abstract: The adzuki bean borer *Ostrinia scapulalis* (Walker) is infected with male-killing *Wolbachia*, which selectively kills male offspring during the embryonic and larval development stages and allows the female offspring survive to adulthood. A high *Wolbachia* density leads to a strong male-killing effect. We utilized rapamycin and 3-methyladenine as an autophagy inducer

and inhibitor to manipulate the autophagy which can change *Wolbachia* density and observed their effects on *Wolbachia* density in larvae and adults of *O. scapulalis*. *Atg8* and *TOR* genes were exploited to predict autophagy activity. The relative density and expression of *Wolbachia*, *Atg8*, and *TOR* were counted by quantitative real-time PCR. We report that the relative density and expression of *Wolbachia* and *TOR* were reduced by rapamycin treatments, whereas the relative expression of *Atg8* was increased in both the larval and adult treatments. The 3-methyladenine treatments exhibited an opposite effect to rapamycin, precisely, relative density and expression of *Wolbachia* and *TOR* were increased and relative expression of *Atg8* was decreased. The female ratio of adults in the larval treatment and offspring in the adult treatments were not affected by the autophagic chemicals. The larval periods were significantly longer and the body weight decreased when the rapamycin was treated to the larvae. The mortality was increased by autophagic chemicals treatment. The abnormality of wing was observed more than normal wing by Rap treatments.

Key words: *male-killing Wolbachia, Atg8, TOR, Ostrinia scapulalis, autophagic chemical*

Introduction

Wolbachia is a gram-negative bacterial endosymbiont infected insects and nematodes that is maternally inherited from mother to offspring (Bandi et al. 2001; Serbus et al. 2008). *Wolbachia* can manipulate various host reproduction functions (Werren et al. 2008), one of which is male killing, a process that selectively kills male offspring during the embryonic and larval development stages and that allows female offspring survive to adulthood (Hurst and Jiggins 2000; Kageyama and Traut 2004; Sakamoto et al. 2007; Sugimoto and Ishikawa, 2012). Male-killing *Wolbachia* occurs in many insects, one of which is the adzuki bean borer *Ostrinia scapulalis* (Walker) (Kageyama and Traut 2004; Sugimoto and Ishikawa 2012), with naturally uninfected females producing male and female offspring in the same ratio, and those who are infected females treated with

tetracycline treatment producing male offspring only (Kageyama and Traut, 2004; Sugimoto and Ishikawa 2012).

Wolbachia density manipulates host reproduction. A higher density of *Wolbachia* leads to strong cytoplasmic incompatibility (CI) in *Laodelphax striatellus* and male-killing in *O. scapulalis*, where the lower density leads to lower CI in *Sogatella furcifera* and a lower female ratio in *O. scapulalis* (Noda et al. 2001; Sugimoto et al. 2015). On the other hand, the *Wolbachia* density is determined by autophagy (Kamalakannan et al. 2015; Voronin et al. 2012), a conserved cell defense mechanism and regulate the target of rapamycin (TOR) of homeostasis (Voronin et al. 2012), which involves the degradation of damaged and unused organelles and proteins and defends against intracellular microorganisms (Levine and Mizushima 2011). Voronin et al. (2012) revealed that the activation of autophagy could reduce the bacterial load to the same extent as antibiotic therapy.

The molecular mechanism of autophagy formation consists of three main steps, namely: 1) autophagy induction, 2) autophagosome nucleation, and 3) autophagosome completion. The lipid-conjugated ubiquitin-like protein *Atg8* gene is localized to autophagosomes and is important for step 3, whereas the *TOR* gene is involved in step 1 (Levine and Mizushima 2011).

Detecting autophagy activity can be achieved by observing the importance of autophagy-related gene expression. *Atg8*, a homolog of the light chain 3 (LC3) protein / γ-aminobutyric acid receptor-associated protein (GABARAP), is one of the most important autophagy-related genes (Nakamura et al. 2022). *Atg8* is planted in autophagic flux as *Atg8*-II, which is converted from *Atg8*-I (Kabeya *et al.*, 2004). There are seven members of the LC3/GABARAP family: LC3A (two splice variants), LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2 (Schaaf et al. 2016; Wang et al. 1999). LC3B or LC3II is the most studied family

protein because it is associated with the maturation of autophagosome flux (Kabeya et al. 2004; Schaaf et al. 2016). Despite each member of the LC3/GABARAP family having a unique role in the execution of autophagy, some of them have also been linked to receptor trafficking (Schaaf et al. 2016).

The *TOR* gene can also be exploited to confirm autophagy activity because *TOR* acts in the opposite direction to autophagy (Kamalakannan et al. 2015; Noda 2017; Raught et al. 2001). *TOR*, a nutrient gene related to growth and aging in eukaryotes, activates anabolic process and represses autophagy (Loewith and Hall 2011; Pazos-solís 2022). *TOR* complex 1 (*TORC1*), a part of *TOR*, regulates various steps of autophagosome formation. Unc-51-like autophagy activating kinase 1 and autophagy-related gene 13 (*Atg13*) are two of the most important substrates of *TORC1* in autophagy (Noda 2017).

Manipulating autophagy is required to reveal the relationship between autophagy and *Wolbachia* and to observe its effect on the sex ratio of *O. scapulalis*. *Wolbachia* density can enhances and suppresses autophagy by employing inducers and inhibitors as autophagic chemicals (Gazali et al. 2022), especially autophagy inducer as rapamycin (Rap) and inhibitor as 3-methyladenine (3-MA) (Poudel et al. 2017; Wang et al. 2016) In this study, the relative density and expression of *Wolbachia*, *Atg8*, and *TOR* were estimated when treated Rap and 3-MA at larval and adult stage. We also investigated the direct effect of autophagic chemicals on the same individual insect when applied as a larval treatment (especially the sex ratio, wing formation, survival ratio, larval periods, and body weight) and to the offspring of treated adults (especially wing formation).

Materials and Methods

Insect rearing

O. scapulalis that were infected and uninfected with male-killing *Wolbachia* were collected in Matsudo, Japan (35.88 N, 139.98 E) in the summer of 2008 – 2009 by Sugimoto and Ishikawa (2012). Pairs of the mated adults were reared in column metal cages (Ø 14 cm × h 15 cm) for two days. Egg colonies were collected in different box cages (Ø 12 cm × h 9 cm) and were separated based on the egg-laying date until the eggs hatch and became larvae, pupae, and adults. All of the insect stages were reared under the conditions of 16L: 8D photoperiod, 55% relative humidity, and 23 °C. Adults were fed 3% sucrose in water, and larvae were fed an artificial diet (Silkmate 2S; Nosan, Japan) (Kageyama and Traut 2004).

Larval Autophagic Chemical Treatment

No more than 24 hours after hatching, *O. scapulalis* larvae that were infected and uninfected with *Wolbachia* were fed with artificial diets mixed with 250 µM and 2 mM of Rap (Funakoshi, Japan) as an autophagy inducer and with 500 µM and 4 mM of 3-MA (Santa Cruz Biotechnology, Inc., USA) as an autophagy inhibitor (10 g: 1ml) from day 1 to day 10 (10d) and along the larval period. The concentration of autophagic chemical was modified based on Gazali et al. (2022). Three groups' replications, with 10 individuals every group of larvae, were separated from each cage and transferred to larger cages (Ø 12 cm × h 9 cm) on day 11, and fed only an artificial diet until they became adults. Three other groups of larvae were also moved into each cage and were fed a diet mixed with autophagic chemicals until they became adults. The number of insect

mortalities were counted manually. Determinations of female ratio and wing formation was conducted by direct observation. The larval period tested consists of three replications, there are 10 individuals in each replication. The body weight of the larvae was determined by analytical balance measurements to 6 individuals on day 11 after hatching.

Adult Autophagic Chemical Treatment

No more than 24 hours after emergence, *O. scapulalis* adults were treated with the same chemicals and concentrations as those used to treat the larvae. *Wolbachia*-infected and uninfected females were put in a plastic cup containing a slice of cotton (l, 1 cm × w, 1 cm) immersed in autophagic chemical solutions mixed with 3% sucrose for three days. Next, pairs of mated adults were reared in metal column cages (Ø 14 cm × h 15 cm) for two days and were fed 3% sucrose in water only which were absorbed with a slice of cotton. Females were moved to a new plastic cup (Ø 12 cm × h 9 cm). One cup was used for one female; then, the adult of treated and untreated groups was collected and stored at freezer –80 °C for DNA and RNA extraction. Determinations of female ratio and wing formation of offspring were conducted by direct observation

DNA, RNA Extraction and cDNA Reverse Transcription

The DNAs from two-thirds of the whole-body posterior of *O. scapulalis* larvae or from two-thirds of the abdominal segment of adults were extracted with the DNeasy Blood and Tissue Kit (Qiagen, Germany) based on the manufacturer's instructions after cleaning the outside of the insect bodies with 70% alcohol. A power masher II machine (Nippi, Japan) was applied to crush the bodies. The DNA concentrations were quantified using a Nanodrop 1000 (Thermo Scientific,

USA). A 260/280 nm absorbance ratio of around 1.8 was acceptable for DNA and cDNA quality (Jasbeer et al. 2009; Taylor et al. 2010). The RNAs were extracted using the Nucleospin RNA isolation kit (Macherey-Nagel, Germany). A power masher II machine (Nippi, Japan) was also applied to crush the remaining one-third of the whole body, excluding the head of the larvae or the remaining segment of the adults as material for RNA extraction. A 260/280 nm absorbance ratio of around 2.0 for the RNAs was acceptable and represented good quality (Taylor et al. 2010). The RNA solutions were stored at −80 °C or were immediately applied for reverse cDNA transcription. cDNAs were reverse transcribed from *O. scapulalis* RNAs using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Japan). The DNA and cDNA solutions were stored at −20 °C or were immediately used in the qPCR reaction.

Primers, PCR, and qPCR

The primers for *Wolbachia surface protein* (*WSP*) to detect *Wolbachia* were chosen based on Werren et al. (1995), and those for WSP-q to quantify *Wolbachia* and EF1α as a reference gene were chosen based on Sugimoto et al. (2015). The *Atg8* degenerate primers were designed from the *Atg8* sequence of the corn borer *O. furnacalis* (Zhang et al. 2018), and the sequenced fragment was used as a material to design the *Atg8*-q primer. *TOR* primers were designed from *TOR*-like sequences, with the accession number DRA014263 for DDBJ, and the sequenced fragment was used as a material to design *TOR*-q primers. All of the primers are listed in Table ESM_1.

Gene detection was conducted by GoTaq (Promega, USA). The reaction volumes are 10 μL of GoTaq, 1 μL of forward and reverse primers, 1 μL of DNA or cDNA templates, and 7 μL sterile purified water (20 μL of total reaction

volume). 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).

A Taqman II reaction mixture was conducted with the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) reagent kit (Takara Bio Inc., JAPAN) for the qPCR reaction. A total of 25 μL of TB Green Premix Ex Taq II, 1 μL of forward and reverse primers, < 100 ng of DNA or cDNA templates, and 8.5 μL of sterile filtered water were used in the reactions. It was run on a Thermal Cycler Dice® Real-Time System II (Takara, JAPAN). The qPCR cycling program for all of the samples was 95°C for 30s, followed by 40 cycles of 95 °C for 5s, and 60 °C for 30s (obtaining the Cq value). Assays were conducted on larvae and adult treatment samples for nine replication which contain three biological replicates with every three technical replicates being used for qPCR reactions. The copy numbers of the *Wolbachia*, *Atg8*, and *TOR* genes were normalized by EF1α (Sugimoto et al. 2015).

Statistical analyses

One-way analysis of variance (ANOVA) followed by Tukey's HSD comparison test was selected to determine the significant differences between treatments. The relative densities and expressions of *Wolbachia*, *Atg8*, and *TOR* were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Larval Treatment

Effect of Autophagic Chemicals on Relative Density of Wolbachia and Relative Expression of Atg8 and TOR.

The effect of autophagic chemicals on the relative density of *Wolbachia* is shown in Fig. 1a. The relative density of *Wolbachia* is significantly reduced by Rap treatment ($p < 0.001$) and is significantly enhanced by 3-MA treatment compared to the control ($p < 0.001$). There was no significant difference between 2 mM and 250 µM ($p = 0.60$) of Rap treatment. There was also no significance difference between 4 mM and 500 µM of 3-MA treatments ($p = 0.90$). But there was significant difference between both of Rap and both of 3-MA treatments ($p < 0.001$).

Fig. 1b shows the effect of autophagic chemicals on the relative expression of *Atg8*. The relative expression of *Atg8* is significantly enhanced by Rap treatment ($p < 0.001$) and is significantly reduced by 3-MA treatment compared to the control ($p < 0.001$). There was no significant difference between 2 mM Rap treatment and 250 µM ($p = 0.99$), and also no significant difference between 4 mM 3-MA treatment and 500 µM ($p = 0.86$), but there was significant difference between both of Rap and both of 3-MA treatments ($p < 0.001$).

The effect of autophagic chemicals on the relative expression of *TOR* is shown in Fig. 1c. *TOR* is reduced by Rap ($p < 0.001$) and is increased by 3-MA ($p < 0.001$) compared to control. In *TOR*, there was no significant difference between 2 mM Rap treatment and 250 µM ($p = 0.94$), and there was no significant difference between 4 mM 3-MA treatment and 500 µM ($p = 0.98$), but there was

significant difference between both of Rap and both of 3-MA treatment ($p < 0.001$).

Effect of Autophagic Chemicals on Larval Period

All of the Rap treatments, except Rap 250 µM at 10d, increased the larval period significantly, whereas the 3-MA treatments have no significantly different compared to control (Fig. 2).

Effect of Autophagic Chemicals on Larval Body Weight.

The autophagic chemicals also affected the larval body weight significantly after 10 days treatment. One-way ANOVA ($F = 15.54$; $df = 29$; $p = 1.65E - 6$) followed by Tukey's HSD shows that Rap treatments both 250 µM and 2 mM slowed the growth of the larvae, whereas 3-MA treatments both 500 µM and 4 mM accelerated it (Fig. 3).

Effect of Autophagic Chemicals on Mortality of Larvae

The mortality of *O. scapulalis* larvae infected *Wolbachia* were significantly difference between all treatments ($p < 0.001$), except between 3-MA 500 µM 10d, 3-MA 4 mM 10d, and 3-MA 500 µM treatments ($p > 0.05$) (Fig. 4).

Effect of Autophagic Chemicals on sex ratio

When treated autophagic chemicals at larval stage, males were never appeared from infected strain (Table 1).

Adult Treatments

Effect of Autophagic Chemicals on Relative Density of Wolbachia and Relative Expression of Atg8 and TOR.

Fig. 5a depicts how autophagic chemicals affect the relative density of *Wolbachia* at adult treatments. Rap treatment substantially lower and 3-MA higher the relative density of *Wolbachia* compared to control ($p < 0.001$). There was no significant difference between 2 mM Rap treatment and 250 µM ($p = 0.57$), and also no significance difference between 4 mM 3-MA treatment and 500 µM ($p = 0.98$), but there was significant difference between both of Rap and both of 3-MA treatments ($p < 0.001$).

The impact of autophagic substances on the relative expression of *Atg8* can be seen in Fig. 5b. In contrast to *Wolbachia*, Rap treatment considerably increases, and 3-MA dramatically decreases the relative expression of *Atg8* as compared to the control ($p < 0.001$). There was no significant difference between 2 mM Rap treatment and 250 µM ($p = 0.98$), and no significance difference between 4 mM 3-MA and 500 µM ($p = 0.6$), but significant difference between both of Rap and both of 3-MA treatment ($p < 0.001$).

The effect of autophagic chemicals on the relative expression of *TOR* is shown in Fig 5c. *TOR* is reduced by Rap treatment and is increased by 3-MA ($p < 0.001$). There was no significant difference between 2 mM Rap treatment and 250 µM ($p = 1.0$), and also no significance difference between 4 mM 3-MA treatment and 500 µM ($p = 0.98$), but significant difference between both of Rap and both of 3-MA ($p < 0.001$).

Effect of Autophagic Chemicals on sex ratio

When treated autophagic chemicals at adult stage, male offspring were never appeared at infected strain (Table. 2).

Discussion

Autophagy can regulate the *Wolbachia* density (Kamalakannan et al. 2015; Strunov et al. 2021; Voronin et al. 2012; Gazali et al. 2022). As an autophagy inducer, Rap enhances the amount Atg8 and inhibits TOR (Beck and Hall 1999; Raught et al. 2001; Takeuchi et al. 2005). Consequently, the *Wolbachia* density was reduced (Kamalakannan et al. 2015; Strunov et al. 2021; Voronin et al. 2012; Gazali et al. 2022). This fact further enriches the reference that autophagy can regulate the density of *Wolbachia* in *O. scapulalis* (Fig. 1a, 5a).

Atg8 was enhanced by the Rap treatments and was lowered by 3-MA compared to control (Fig. 1b, 5b). This fact shows that as an autophagy inducer and inhibitor, Rap and 3-MA treatments have been successful in regulating autophagy, as determined by observing the relative expression of *Atg8*. *TOR* gene expression was reduced and enhanced by the Rap and 3-MA treatments respectively (Fig. 1c, 5c). On the other hand, 3-MA treatment reduced *Atg8* expression (Fig. 1b, 5b), which was accompanied by an increase in *TOR* expression. This fact shows that the autophagic chemicals treatments have successfully confirmed that the TOR and autophagy mechanisms in *O. scapulalis* are in opposite directions. The presence of TOR, which activates the anabolic process, is in the opposite direction in the presence of autophagy (Pazos-solís 2022). Rap enhanced the expression of *Atg8*, which was accompanied by a decrease of *TOR* (Loewith and Hall 2011).

Based on Figures 1 and 5, the relative densities of *Wolbachia* are in different patterns compared to *Atg8*, but it is in line with the relative expression of *TOR* in both larvae and adult treatment. It can be concluded that *Wolbachia* does not interfere with the nutritional system of *O. scapulalis* and that *TOR* signaling is more active in the presence of nutrients (Crespo and Hall 2002; Raught et al. 2001).

The autophagic chemicals treatment on *O. scapulalis* larvae also affect the body weight. Figure 3 shows the larval body weight decreased after they were treated with Rap for 10 days. In contrast, the larvae gained body weight after they were fed with 3-MA for 10 days. These results inform that Rap and 3-MA stunt and fuel the larval growth. This finding corroborates the previous study by Scieuzo et al. (2018) which found that Rap treatment to larvae reduced the pupa weight in *Heliothis virescens*. They speculate, Rap has a modulatory effect that influences the development of all tissues.

Based on the Figure 2, it can be understood that the 250 µM Rap treatment along the larval stages prolongs the larval stage period more than the 10d treatment. Treatment of Rap 2 mM for 10d was not different from that of during the larval phase, we suspect that this was caused by only one insect survived as long as the treatment of 2 mM Rap during the larval phase. Rap treatments in sort time sometimes has no effect on the length of the insect's larval period, this is confirmed by Gazali et al. (2022) that treating Rap for 3 days to *L. striatellus* did not increase the length of insect life span.

In previous studies, *Wolbachia* kill the male and leave the female offspring in *O. scapulalis* (Kageyama and Traut 2004; Sugimoto and Ishikawa 2012). Decreasing the density of *Wolbachia* by tetracycline hydrochloride and a short high temperature treatment within *O. scapulalis* larvae led to the emergence of

male offspring (Sugimoto et al. 2015). Even though utilizing Rap reduced *Wolbachia* density (Fig. 1a, 5a), but no male insects were observed on the new emerging adults (Table 1, 2). We suspect that autophagic chemicals did not affect the sex ratio. It is important to do further study, considering that the method of utilizing autophagic chemicals reduces *Wolbachia* as same as the previous method (Sugimoto et al. 2015), but has a different effect on sex ratio, and regarding the possibility that not only *Wolbachia* has an effect on the sex ratio but there are other factors such as the transformer gene (Bopp et al. 2014), virus (Fujita et al. 2021) or other symbionts (Kageyama et al. 2012) that are currently not conclusive, it is important to conduct additional research.

The abnormal wings were observed often than normal wings in both larvae and adult treated with Rap compared to control, but not by 3-MA (except by 3-MA 500 µM and 4 mM on larvae infected *Wolbachia*) (Table ESM_2, ESM_3). This fact suggests that the Lepidopteran insect has a different sensitivity to Rap.

Rap and 3-MA also seems to influence the mortalities of larvae (Fig. 4), the more or the longer of Rap and 3-MA utilized the higher the mortality of the larvae compared to the control. Larval mortalities with Rap treatment were also higher than the 3-MA treatment (Fig 4). All the mentioned conditions make us suspect that the concentration of autophagic chemicals used was too high to develop, especially for Rap concentration. This fact further supports the allegation that the current of Rap concentrations have had a toxic effect on *O. scapulalis,* whereas Rap has been used at higher doses in *H. virescens* by Scieuzo et al. (2018).

Disclosure

All authors have seen and agree with the contents of the manuscript and there is no conflict of interest, including specific financial interest and relationships and affiliations relevant to the subject of manuscript.

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Fig. 1 Effect of autophagic chemicals treatment to relative densities of *Wolbachia* and relative expression of *Atg8* and *TOR* within *O. scapulalis* larvae (A) Relative densities of *Wolbachia.* (B) Relative expressions of *Atg8*. (C) Relative expressions of *TOR*. Vertical bar indicates the ± of SE. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Fig. 2 Comparison of larval period of *O. Scapulalis* infected *Wolbachia* with after autophagic chemical treatment for ten days (10d) and along the larval period. Vertical bar indicates the ± of SD. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Fig. 3 Comparison of larval body weights after autophagic chemicals treatment. Vertical bar indicates the ± of SD. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Fig. 4 Comparation of larval mortalities after autophagic chemical treatment for ten days (10d) and along the larval period. Vertical bar indicates the ± of SE. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Fig. 5 Comparison of relative densities and gene expressions between treatments on *O. scapulalis* adults. (A) Relative densities of *Wolbachia.* (B) Relative expression of *Atg8*. (C) Relative expression of *TOR*. Vertical bar indicates the ± of SE. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Table 1. Female ratio of *O. scapulalis* after treated by autophagic chemicals (larvae treatment)

Table 2. Female ratio of offspring of *O. scapulalis* after treated by autophagic chemicals (adult treatment)

Figure1

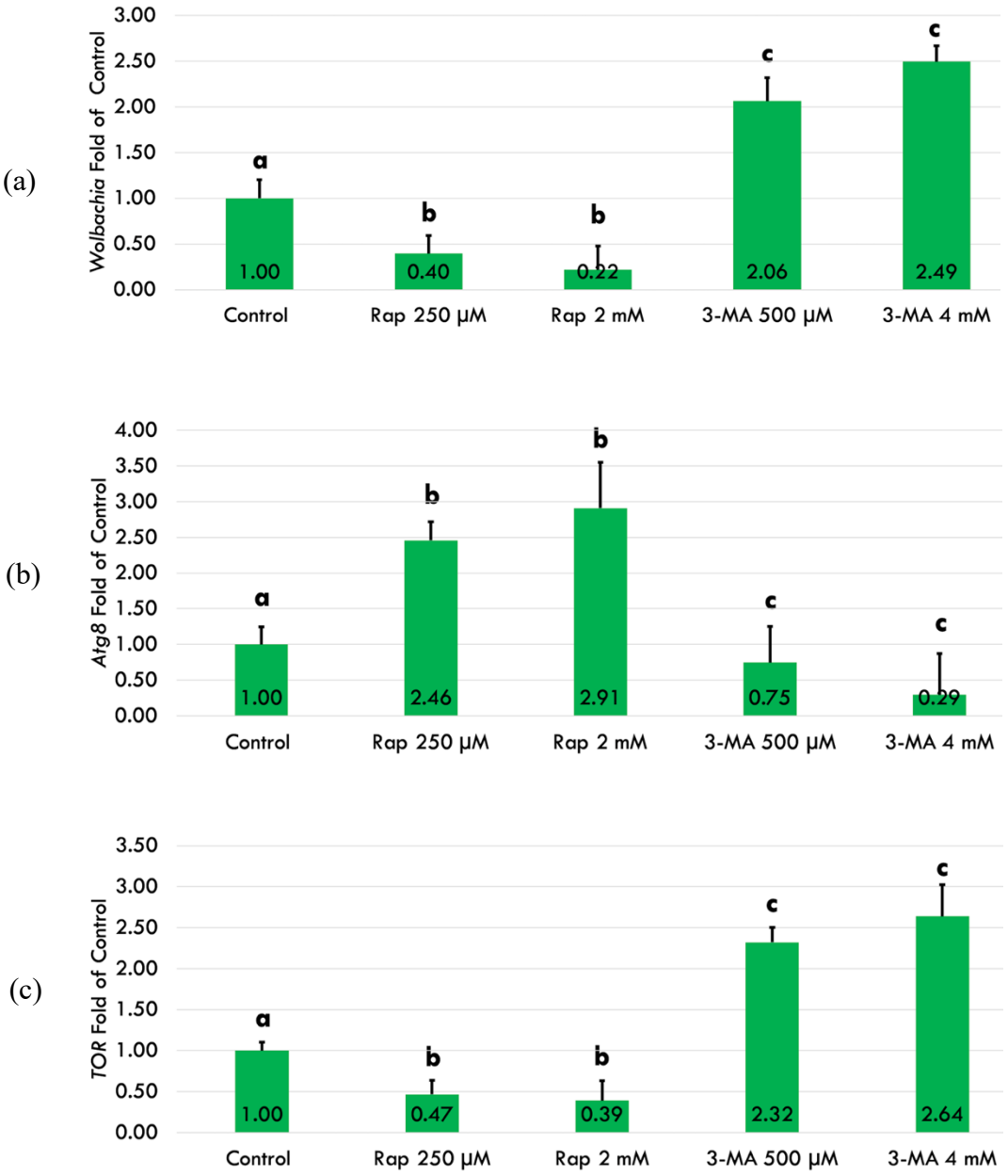

Fig. 1 Effect of autophagic chemicals treatment to relative densities of *Wolbachia* and relative expression of *Atg8* and *TOR* within *O. scapulalis* larvae (A) Relative densities of *Wolbachia.* (B) Relative expressions of *Atg8*. (C) Relative expressions of *TOR*. Vertical bar indicates the ± of SE. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Figure2

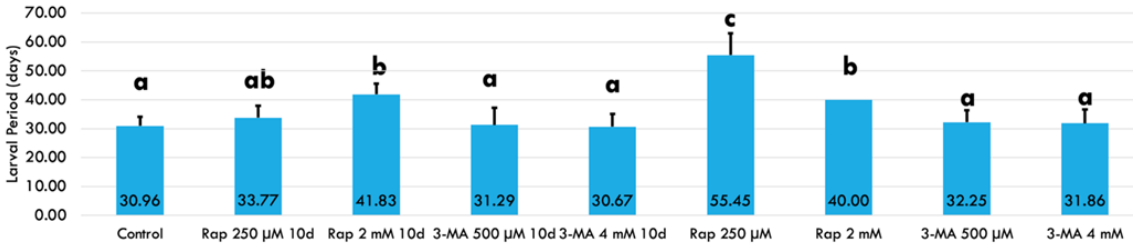

Fig. 2 Comparison of larval period of *O. Scapulalis* infected *Wolbachia* with after autophagic chemical treatment for ten days (10d) and along the larval period. Vertical bar indicates the ± of SD. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Figure3

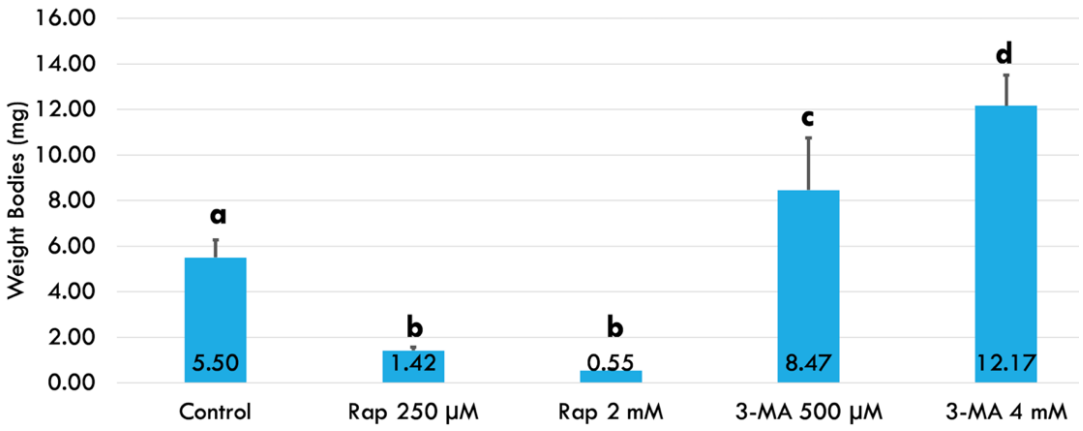

Fig. 3 Comparison of larval body weights after autophagic chemicals treatment. Vertical bar indicates the ± of SD. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Figure5

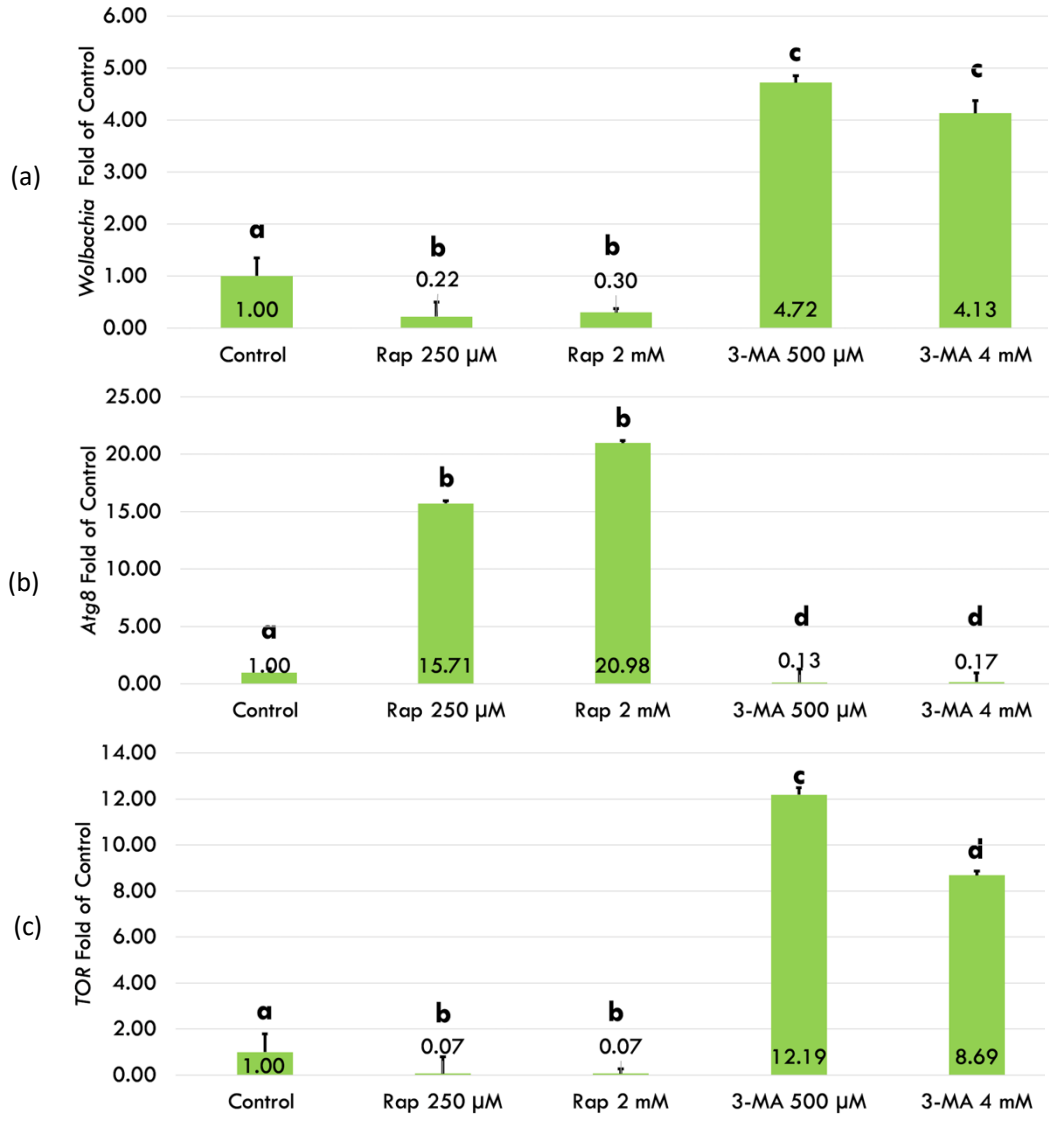

Fig. 5 Comparison of relative densities and gene expressions between treatments on *O. scapulalis* adults.

(A) Relative densities of *Wolbachia.* (B) Relative expression of *Atg8*. (C) Relative expression of *TOR*.

Vertical bar indicates the ± of SE. The significant differences among the treatments are indicated by

different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Figure4modified

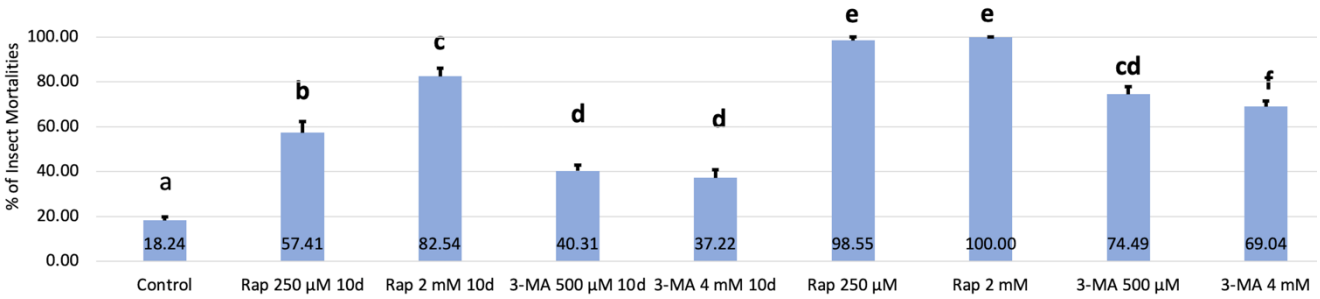

Fig. 4 Comparation of larval mortalities after autophagic chemical treatment for ten days (10d) and along the larval period. Vertical bar indicates the ± of SE. The significant differences among the treatments are indicated by different letters (one-way ANOVA $p < 0.01$ followed by Tukey's comparison test).

Table 1. Female ratio of *O. scapulalis* after treated by autophagic chemicals (larvae treatment)

Treatment	Uninfected	Infected
Control	50.00% (60)	100% (25)
Rap 250 μM 10d	23.07% (13)	100% (26)
Rap 2 mM 10d	66.67% (6)	100% (17)
3MA 500 μM 10d	51.61% (31)	100% (25)
3MA 4 mM 10d	45.00% (20)	100% (10)
Rap 250 μM	100% (1)	100% (6)
Rap 2 mM	0%	100% (3)
3MA 500 μM	20.00% (15)	100% (14)
3MA 4 mM	55.56 % (18)	100% (10)

Parenthesis indicates no. of tested individuals

Table 2 Female ratio of offspring of *O. scapulalis* after treated by autophagic chemicals (adult treatment)

Treatment	Uninfected	Infected
Control	52.34% (428)	100% (109)
Rap 250 μM	55.56% (9)	100% (57)
Rap 2 mM	42.11% (19)	100% (14)
3MA 500 μM	51.63% (306)	100% (126)
3MA 4 mM	50.08% (311)	100% (122)

Parenthesis indicates no. of tested individuals

ESM1

Supplementary MaterialmodifiedESM_2