Venom components of *Asobara japonica* impair cellular immune responses of host *Drosophila melanogaster*

Running heading: *Asobara japonica* venom impairs host cellular immunity

Shunsuke X. Furihata[1], Hitoshi Matsumoto[2], Masahito T. Kimura[3], and Yoichi Hayakawa*[1, 2]

[1]The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan; [2]Department of Applied Biological Sciences, Saga University, Saga 840-8502, Japan; [3]Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

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* *Correspondence*: Yoichi Hayakawa, Department of Applied Biological Sciences, Saga University, Honjo-1, Saga, 840-8502 Japan.

Tel/Fax:81 952 28 8747; E-mail: hayakayo@cc.saga-u.ac.jp

Abstract

The endoparasitoid wasp *Asobara japonica* has highly poisonous venom: the host *Drosophila* larvae are killed by envenomation at a dose that is naturally injected by the female wasp at parasitism. This insecticidal venom is neutralized, however, because *A. japonica* introduces lateral oviduct components soon after venom injection at oviposition. Although the venom and lateral oviduct components of this parasitoid have been partially characterized, how the venom components favor successful development of wasp eggs and larvae in the host remains ambiguous. Here we demonstrated that *A. japonica* venom did not affect host humoral immune responses, determined as expression of antimicrobial peptide (AMP) genes, but significantly diminished two cellular responses, spreading and phagocytosis, by host hemocytes. Moreover, venom components drastically elevated a serine protease-like activity four hours after its injection. The lateral oviduct components did not negate the detrimental effects of the venom on host cellular immunities but significantly reduced the venom-induced elevation of protease activity. Both active factors in venom and lateral oviduct components were roughly characterized as heat-labile substances with a molecular mass of at least 10 kDa. Finally, venom of *A. japonica*, with a wide host range, was found to be much more toxic than that of *A. rossica*, which has a limited host range. These results reveal that *A. japonica* venom toxicity allows exploitation of a broader range of host insects because it is essential to overcome cellular immune responses of the host for successful parasitism.

Keywords: Parasitoid wasp; *Drosophila*; cellular immunity; lateral oviduct; venom.

Introduction

Endoparasitoid wasps parasitize other arthropods (largely insects) by ovipositing into their hosts (Moreau and Guillot, 2005; Vinson and Iwantsch, 1980). After parasitism, the host's physiology is altered to support parasite development. Parasitoid-host compatibility is defined by the balance between the parasitoid's ability to invade the host and the host's ability to eliminate the parasitoid (Eslin and Prevost, 2000; Strand and Pech, 1995). A parasitoid must both satisfy its developmental requirements and avoid elimination by the host's immune system. The host insect's immune system serves as a key defense against attack by parasitoids. Insects have a well-developed innate immune system that is often divided into humoral and cellular components for convenience of discussion (Hetru et al., 2003; Strand, 2008; Williams, 2007). Among the humoral effector molecules are the antimicrobial peptides (AMP) that kill bacterial and fungal pathogens. Cellular immune responses refer to defense responses, like phagocytosis and encapsulation, that are mediated by hemocytes. Even though host insects possess effective defense mechanisms that protect them from intrusions, parasitoid wasps have evolved a variety of strategies for compromising or avoiding host immune responses, and thus parasitoids do not fail to overcome the host immune system if they have the natural parasitoid-host compatibility.

The strategies of endoparasitoid wasps for overcoming host immunity can be divided into passive and active mechanisms (Moreau et al., 2003; Schlenke et al., 2007). Passive mechanisms include developing in locations that protect the parasitoid from encapsulation or possessing surface features that prevent the host from recognizing the parasitoid as non-self. It is known that a few endoparasitoid wasps avoid encapsulation by developing in organs like nerve ganglions, where they are protected from circulating host hemocytes (Leiby and Hill, 1923). Some parasitoids also avoid encapsulation by attaching on host tissues with their sticky chorion (Prevost et al., 2005; Schlenke et al., 2007). Most endoparasitoids, however, develop in the host hemocoel where they are fully exposed to circulating hemocytes. Endoparasitoid eggs and larvae are often covered by surface factors that are either not recognized as foreign or to which hemocytes do not adhere (Hayakawa and Yazaki, 1997; Tanaka et al., 2002). Active mechanisms refer to a parasitoid disrupting one or more distinct functions of the host immune system. These require delivery of genes for synthesis of active proteins *in situ* (polydnaviruses, teratocytes) and/or proteins (venoms or ovarian proteins) from the

female reproductive tract (Gopalapillai et al., 2005; Luckhart and Webb, 1996; Richards and Parkinson, 2000). Although polydnaviruses are among the most studied examples of effectors introduced by parasitoids and have been reported to contribute both active and passive immune inhibitory mechanisms, they have been found only in parasitoid wasps that parasitize lepidopteran host insects (Burke and Strand, 2012; Summers and Dib-Hajj, 1995).

Asobara japonica is a generalist endoparasitic braconid wasp that attacks a number of distantly related hosts. However, the habitual host range is limited within Drosophilidae and *A. japonica* does not carry polydnavirus. Even though *A. japonica* and a few wasp species in the same genus do not have this well-known effector factor (Eslin et al., 1996), they successfully parasitize *Drosophila* by means of various strategies: the parasitoid *A. citri* actively inhibits the cellular immune defenses of the host, while *A. tabida* successfully parasitizes without modifying the encapsulation capacity of host hemocytes (Moreau et al., 2003). Furthermore, interruption of the oviposition behavior of *A. japonica* induced the death of the host insect at extremely high rates (Furihata and Kimura, 2009; Mabiala-Moundoungou et al., 2010). The high mortality of the hosts was demonstrated to be due to venom components introduced into the host insects by the interruption of oviposition. Furthermore, substance(s) provided by the female wasp or ovarian extracts from female wasps suppressed the insecticidal effect of venom on the host (Mabiala-Moundoungou et al., 2010). However, the physiological roles as well as the molecular features of both components, the venom toxin and oviduct antitoxic substance, remain ambiguous. In the present study, we tried to clarify these uncertainties by means of physiological techniques using several related species of parasitoid wasps and host *Drosophila* species.

MATERIALS AND METHODS

Insects

Drosophila ficusphila and *D. auraria* were collected in Iriomote-jima and Sapporo, Japan, respectively. *D. ananassae* was supplied by Fly Stock of Kyorin University. All of these flies were reared on an artificial diet at 23±1^{o}C, 15:9 L:D photoperiod (Furihata and Kimura, 2009). *Asobara japonica* was collected in Tokyo, Japan and reared in *D. simulans* or *D. melanogaster* as hosts, while *A. rossica*, originated from Sapporo, Japan,

was reared in *D. auraria* (Ideo et al., 2008). All parasitoids were maintained under the same conditions of light and temperature as the host strains. Interruption of oviposition behavior (Interrupted Parasitism) was performed according to the procedure as previously described (Furihata and Kimura, 2009).

Microinjection of venom with/without ovarian fluid into Drosophila larvae

Venom reservoirs and lateral oviducts were dissected from *Asobara* wasp females and were separately put into a drop of chilled phosphate buffered saline (PBS: 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.2). Venom reservoirs were lightly homogenized, lateral oviducts were squeezed with fine forceps, and centrifuged at 15,000 rpm for 15 min at 4^{o}C to collect the supernatants. Venom supernatant (V) or mixture of venom and lateral oviduct supernatants (V+O) were diluted up to 15 µl per a female with PBS. Six-day-old (third instar) larvae of *Drosophila* were injected with 0.15 µl of each sample unless otherwise stated, put on diet medium, and survival rates were measured at 24 h after injection.

Quantitative RT-PCR of AMP genes in D. melanogaster larvae

To determine whether the *Drosophila melanogaster AMP* genes are expressed in larvae, Quantitative RT-PCR was conducted essentially according to the procedure described previously (Ninomiya et al., 2008). First-strand cDNA was synthesized with oligo$(dT)_{12\text{-}18}$ primer using ReverTra Ace RT-PCR kit (Toyobo) according to the manufacturer's protocol. The copy numbers of RNA encoding the genes of interest were standardized against that of the RNA coding *rp49* in each sample. Specific primer pairs of *Drosophila AMP*s are as follows. *Drosomysin*: TTGTTCGCCCTCTTCGCTGTCCT and GCATCCTTCGCACCAGCACTTCA,
Metchnikowin: ATTCCCGCCACCGAGCTAA and CAATGTGTTAACGACATCAGC,
Attacin: GTGGTGGGTCAGGTTTTCGC and TGTCCGTTGATGTGGGAGTA,
Diptericin: GTTCACCATTGCCGTCGCCTTAC and CCCAAGTGCTGTCCATATCCTCC,
rp49: AGATCGTGAAGAAGCGCACCAAG and CACCAGGAACTTCTTGAATCCGG.

In vitro morphological analysis of Drosophila larval hemocytes

Whole hemolymph was collected from test *Drosophila* larvae into a 10 µl drop of chilled Schneider's *Drosophila* medium containing each indicated sample plus 10 nM *Drosophila* growth-blocking peptide (dGBP) (Tsuzuki et al., 2012) and incubated at 25^{o}C for 1 h and cells were observed by using laser scanning confocal microscope (Ez-T1 system, Nikon, Japan). Cells were scored as spread if they assumed a flattened morphology and were ≥ 24 µm along their longest axis (Oda et al., 2010). For measuring phagocytotic activities, 2 µl suspension of fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* was added, and incubated for another 1 h at 25^{o}C in dark place. After nuclear staining with Hoechist 33258, hemocytes was washed with anti-coagulant buffer (41 mM citric acid, 98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, pH 4.5) and resuspended in 10 µl Schneider's medium containing 100 µg/ml ethidium bromide (EtBr). Under this condition, cells phagocytized FITC-labeled *E. coli* showed a bright green fluorescence. Live and dead cells appeared blue and dark red in color, respectively.

Measurement of protease-like activity using synthetic substrate-MCA

The release of 7-amino-4-methylcoumarin from synthetic peptide-MCA substrate during the hydrolase reaction was detected using a multimode detector DTX800 (Beckman Coulter) as described previously (Hayakawa, 1994); the hydrolase activity was regarded as a protease-like activity of each sample. Hemolymph was collected from test *Drosophila* larvae into a 50 µl drop of chilled PBS containing 0.1% *N* -phenylthiourea (PBS-PTU), immediately centrifuged at 2,000rpm for 3 min at 4^{o}C, and supernatant and pellet were collected to use as plasma and hemocyte fractions, respectively. After sonication of hemocyte suspension in PBS-PTU and subsequent centrifugation at 15,000 rpm for 5 min at 4^{o}C, the supernatant was referred to as a hemocyte lysate sample. Five µl of each sample was incubated in 100 µl of 10 mM Tris-HCl buffer solution (pH 7.8) containing 20 µM peptide-MCA at 30^{o}C for 1 h. After adding 100 µl of 50% acetic acid to the reaction mixture, the fluorescence of the mixture was measured.

Statistical analyses

Means were compared with separate one-way analysis of variance (ANOVA) using Excel 2010 (Microsoft Corp., 2010) and discriminated at Type I error = 0.05.

RESULTS

Physiological effects of A. japonica venom components on D. melanogaster

Prior studies indicated that solely venom components are introduced into the host larvae when oviposition by the *A japonica* female is interrupted a few seconds after insertion of the ovipositor, but it causes 98% mortality in the host *Drosophila simulans* larvae (Furihata and Kimura, 2009). This is true, also, for a different host, *D. melanogaster* (Fig. 1A). Contrarily, when the wasp completed normal oviposition behavior, the host larvae were injected with eggs together with lateral oviduct secretory components following venom injection, and most of the host larvae were sacrificed by successful parasitism: adult wasps emerged from 86 % of the parasitized hosts (Fig. 1A). The insecticidal effect, followed by interrupted oviposition, was reproduced by injection of venom components prepared from the venom reservoir of female wasps (Fig. 1B). The venom-induced insecticide ability was neutralized by the lateral oviduct extract, and both venom toxicity and oviduct antitoxicity were found to be heat labile (Fig. 1B). The insecticidal activity of the venom components was dose-dependent: only 0.0075 female equivalent amounts of venom components were required to kill over 90% of *D. melanogaster* larvae (Fig. 1C). Furthermore, the survival rates of *D. melanogaster* larvae decreased almost linearly up to 12 hours after the injection of 0.003 female equivalent amounts of the venom components (Fig. 1D). These data are entirely consistent with those when previously obtained with *A. japonica* and another habitual host *D. simulans* (Furihata and Kimura, 2009). We next examined the effects of *A. japonica* venom components on larvae of the non-habitual host *D. ficusphila.* The results showed the species-specific activity of venom components: injection of the venom components did not have any detrimental effects on *D. ficusphila* larvae (Fig. 1B).

To roughly characterize the molecular mass of the venom toxic factor, we used two kinds of centrifugal filters (Millipore Co.), and found that the toxic factor passed through the 0.22-μm pore filter (V-0.22) but not through the 10 kDa MW cut-off filter

(V-10kDa): the filtrates that passed through the V-0.22 filter and V-10kDa filter produced, respectively, over 90% and less than 4% mortality on the test larvae (Fig. 1E). These data indicated that the insecticidal factor is a heat-labile substance with a molecular mass larger 10 kDa.

Effects of A. japonica venom toxic factor on D. melanogaster immunity

We next examined whether *A. japonica* venom components affect expression of humoral effecter molecules such as antimicrobial peptides (AMP) of the host larvae because a variety of detrimental effects on host insect immunities following parasitism by a parasitoid wasp have been reported (Schmidt et al., 2001). Although expression of all tested *AMP* genes, *Drosomicin, Metchnikowin, Attacin*, and *Diptericin*, was increased by injection of every sample including the control PBS, there was no significant difference between expression levels in larvae injected with PBS and venom components (or venom together with lateral oviduct components) (Fig. 2), indicating that neither venom nor ovarian components affected expression of humoral immune responses such as *AMP* gene expression.

We next examined the effects of *A. japonica* venom components on the cellular immune responses of host larvae. After hemocytes isolated from *D. melanogaster* larvae were incubated with venom components, we observed the hemocytes by confocal microscopy to measure their survival rates (Fig. 3A). Exposure of hemocytes to venom components significantly diminished their survival rates: the exposure to the medium (Schneider's *Drosophila* medium), venom components, venom components with lateral oviduct extract, and lateral oviduct extract alone induced 87%, 60%, 59% and 87% survival rates, respectively (Fig. 3B), indicating that venom components significantly decreased the survival rates of hemocytes but that the oviduct components were unable to neutralize the venom effect.

The effects of venom components on spreading responses of *D. melanogaster* hemocytes were examined. Co-incubation of test hemocytes with the venom components significantly disturbed normal spreading responses of hemocytes (Fig. 4A). Furthermore, pretreatment of *D. melanogaster* hemocytes with the venom components significantly diminished phagocytotic rates of test hemocytes engulfed by *E. coli* from 25% to only 1% (Fig. 4B). This decline in the phagocytotic activity of test hemocytes was not rescued by addition of lateral oviduct components. These results strongly

indicated that the venom toxic factor(s) largely diminished cellular responses of the host hemocytes but that lateral oviduct components were unable to neutralize the disturbance. The effects of *A. japonica* venom components on hemocytes prepared from *D. ficusphila*, a non-habitual host species of *A. japonica*, were examined. Neither survival rates nor phagocytic activities of *D. ficusphila* hemocytes were significantly changed after treatment with the *A. japonica* venom factor (data not shown), indicating that *A. japonica* venom toxin-induced disturbance of cellular immunity is species-specific.

Protease activity in hemolymph of venom-injected larvae

The fact that exposure of *D. melanogaster* larval hemocytes to venom components significantly decreased their survival rates enabled us to presume that proteolytic activities would be elevated in the hemolymph via the venom-induced cell lysis. We measured proteolytic activities in plasma (devoid of cells) of *D. melanogaster* larvae using ten synthetic peptide-MCA substrates after injection of *A. japonica* venom components (Fig. 5A). The results indicated that the hydrolytic activity of one substrate (Boc-Ile-Glu-Gly-Arg-MCA) was increased specifically following the venom component injection (Fig. 5A, B). Characterization of the proteolytic activity profile of the hemocyte lysate using the same ten peptide substrates revealed an evident difference between the patterns of plasma and hemocyte lysate enzyme activities (Fig. 5A and 5C), indicating that the venom-induced proteolytic activity in the plasma is not directly derived from hemocyte lysate but rather must be caused via a certain chain reaction triggered by the venom-induced cell lysis. The plasma proteolytic activity was time-dependently elevated and reached to an almost 300-fold level at four hours after venom injection. This increase was completely blocked by co-injection of the lateral oviduct extract (Fig. 5B). This neutralizing effect was diminished by heating the lateral oviduct preparation at 100^{o}C for five minutes (data not shown), which is very similar to the heat-lability of the neutralizing activity of the oviduct factor against the insecticidal effect by the venom components as shown in Fig. 1B. No elevation of proteolytic activity induced by venom toxin was observed in the plasma of *D. ficusphila* injected with the *A. japonica* venom components, indicating that the *A. japonica* venom-induced increase in the proteolytic activity is species-specific (Fig. 5D).

Comparison of venom toxicities of A. japonica and A. rossica

A. japonica adopts generalist strategies that exploit a number of more distantly related host species. In contrast, *A. rossica* is regarded as a specialist because *D. auraria* has been regarded as their only habitual host so far known (Mitsui et al., 2007). We first tested parasitoid-host compatibilities of both wasps and *Drosophila* host candidate species. After oviposition by *A. japonica* of *D. ananassae* and *D. auraria*, wasps emerged from over 60% of both test hosts, indicating that *A. japonica* successfully utilized both *Drosophila* species as habitual hosts (Fig. 6A). However, after oviposition by *A. rossica* of *D. melanogaster and D. auraria*, wasps successfully emerged from 45% of parasitized *D. auraria*, while no wasp emerged from parasitized *D. melanogaster* (Fig. 6B). Furthermore, interruption of the oviposition behavior of *A. japonica* killed most *D. ananassae* and *D. auraria* larvae, while interruption of the *A. rossica* oviposition behavior did not kill any *D. melanogaster* or *D. auraria* larvae (Fig. 6A), implying that venom components of *A. rossica* may not have any insecticidal effect against either *Drosophila* species. This speculation was completely confirmed by comparison of venom toxicity between *A. japonica* and *A. rossica*. While injection of *A. japonica* venom killed most of *D. ananassae* and *D. auraria* larvae, injection of *A. rossica* venom killed neither *D. melanogaster* nor *D. auraria* larvae (Fig. 6C, D). These results implied the possibility that highly toxic venom components of *A. japonica* have long contributed to exploitation of a broader range of *Drosophila* species as hosts during the evolutionary process.

DISCUSSION

We emphasize the unique parasitic strategy of *A. japonica*: the wasp injects highly poisonous venom components prior to the introduction of an egg together with neutralizing soluble components of the lateral oviducts during every oviposition behavior. Quantitative analysis of the toxicity of *A. japonica* venom enabled us to conclude that the wasp venom components exert a strong toxic activity against the host *D. melanogaster* larvae. The LD_{50} calculated from the dose-response curve was only 0.001 female-equivalents per host. Since it has been reported that another braconid wasp, *Microplitis demolitor,* injects 0.01-0.02 female-equivalents/host of calyx fluid (lateral oviduct soluble fraction) at each oviposition, this LD_{50} value, 0.001 female-equivalents per host, is a dose much lower than that injected by the wasp during

each oviposition event even under physiological conditions (Strand and Dover, 1991). We infer that the amount of venom components injected by *A. japonica* at every normal parasitism is high enough to kill the host larva. Although it has been reported that some ectoparasitoid species possess highly poisonous venoms, such toxic venom is rare in endoparasitoids (Er et al., 2011; Rivers et al., 1993).

The toxicity of *A. japonica* venom is species-specific: the venom was highly poisonous to the habitual hosts, *D. melanogaster, D. ananassae, D. auraria*, and *D. simulans*, while not at all poisonous to the non-habitual host *D. ficusphila*. The correlation between the venom-sensitivity and parasitoid-host compatibility led us to presume that the highly toxic venom disturbs a normal defense mechanism of the host. Based on this speculation, we first examined *AMP* gene expression levels, but the tested gene expression levels were not significantly different following injection of the venom components. In contrast, the survival rates and cellular immune response activities significantly declined during incubation of *D. melanogaster* hemocytes with *A. japonica* venom. In particular, spreading and phagocytosis activities of hemocytes were abruptly decreased by co-incubation with the venom. We take this to mean that the cellular immune responses of host hemocytes are impaired by venom components. The fact that the injection of venom components precedes the oviposition during natural parasitism is consistent with our speculation that avoidance of the normal host defense mechanism is essential for survival of the parasitoid eggs in the host hemocoel (Kraaijeveld and Godfray, 2009; Prevost et al., 2009; Vinson and Iwantsch, 1980).

We found a remarkable elevation of the serine protease-like activity against the synthetic peptide-MCA substrate (Boc-Ile-Glu-Gly-Arg-MCA), which is used to monitor the activity of Factor Xa *in vitro* (Morita et al., 1977), in *D. melanogaster* larval plasma following injection of *A. japonica* venom. The hydrolytic activity of this peptide-MCA substrate does not have the highest esterase activity in the lysate of *D. melanogaster* hemocytes, suggesting to us that the increase in the plasma protease-like activity is not directly derived from the venom-induced lysis of hemocytes. Elevation of the protease-like activity did not occur in the plasma of *D. ficusphila* larvae injected with *A. japonica* venom, indicating that *D. ficusphila* hemocytes are insensitive to *A. japonica* venom. This interpretation was supported by the fact that survival rates as well as phagocytosis activities of *D. ficusphila* hemocytes were not affected by co-incubation with *A. japonica* venom components. The venom-induced protease-like enzyme

activation in the *D. melanogaster* plasma is completely blocked by co-injection with components of the lateral oviduct. Therefore, we conclude that the venom-induced activation of the protease-like enzyme must be directly or indirectly related to the insecticide ability of the venom components because this blockage by the oviduct components shares a striking similarity with the neutralization of the venom-induced insecticidal effect. The toxic factor(s) in *A. japonica* venom were roughly characterized as a heat-labile substance(s) with a molecular mass larger than 10 kDa. Although, at present, we do not have direct evidence supporting the possibility that this factor directly binds to *D. melanogaster* hemocytes and induces the lysis, it is possible that a cascade reaction of plasma protease activities triggered by the venom toxic factor(s)-induced lysis of hemocytes inactivates and/or eliminates normal hemocytes. Characterization of the functional role as well as the molecular nature of the *A. japonica* venom toxic factor(s) are subjects that deserve further investigation.

Antitoxic factor(s) introduced immediately after injection of the venom toxin are crucial for successful parasitism by *A. japonica* wasps. The mechanism by which antitoxic factor(s) derived from the lateral oviducts of the wasps neutralize the venom insecticidal factor(s) is unknown. However, the two following observations in the present study denote the target process of the factor. 1) The antitoxic factor(s) were unable to repress the venom toxin-induced decrease in the survival rates and phagocytosis activities of *D. melanogaster* hemocytes. 2) However, this antitoxic factor(s) enabled complete inhibition of the venom toxin-induced elevation of the plasma protease-like activity. These observations tell us that the oviduct components target not the process of the venom-induced hemocyte damage but the process of activation of the protease-like enzyme in the plasma. The fact that the lateral oviduct also enables neutralization of the insecticidal effect of the venom toxin also led us to speculate that the venom-induced activation of the serine protease-like activity in plasma might have a direct or indirect relationship with the insecticide ability of the venom components.

A. rossica is reported to be a close relative of *A. japonica*, but the host ranges of these species are completely different: the former can parasitize only *D. auraria*, but the latter can utilize more distantly related hosts (Ideo et al., 2008). Therefore, it is reasonable to regard *A. rossica* and *A. japonica* as a specialist and a generalist parasitoid, respectively. Comparison of the effects of venom components prepared from both wasp

species on *Drosophila* species confirmed that *A. japonica* venom killed all tested *Drosophila* larvae but *A. rossica* venom did not kill larvae of its own habitual host, *D. auraria*, or *D. melanogaster*. Based on the results of these comparative experiments, it is reasonable to propose that the toxicity of venom components has a consequential impact on the host range of the *Asobara* parasitoid family.

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Figure Legends

Figure 1. Effects of parasitism by *Asobara japonica* and its venom injection on *Drosophila* larvae. (A) Percentages of *D. melanogaster* larvae that died at the larval or pupal stage (black), survived until eclosion (white), and produced adult wasp (gray) after uninterrupted (U) or interrupted (I) parasitism by *A. japonica*. (B) Survival rates of *D. melanogaster* and *D. ficusphila* larvae injected with PBS, *A. japonica* venom (V), the boiled venom (bV), the venom with the lateral oviduct components (V+O), or the venom with the boiled lateral oviduct components (V+bO). Survival rates were measured 24 h after injection of each sample. Data are given as means±SE for seven separate measurements using 6-7 larvae each. Significant difference between treatments was detected (*$P < 0.05$). (C) Effect of various concentrations of *A. japonica* venom on survival rates of *D. melanogaster* larvae. Data are given as means±SE for five separate measurements using 6-7 larvae each. Significant difference was detected relative to value without venom. Other explanations are as in (B). (D) Effect of time after injection of 0.003 female equivalent amounts/larva venom on survival rates of *D. melanogaster* larvae. Data are given as means±SE for three separate measurements using 5-10 larvae each. Significant difference was detected relative to value at 0 h. Other explanations are as in (B). (E) Survival rates of *D. melanogaster* larvae injected with residue (Rs) or filtrate (Ft) of *A. japonica* venom after filtration through 0.22μm-pored filter (V-0.22) or 10kDa cut-off filter (V-10kDa). A filtrate residue (Rs) left in the filter media was reconstituted with the original volume of PBS and injected into test larvae. Data are given as means±SE for five separate measurements. Other explanations are as in (B).

Figure 2. Relative expression of four antimicrobial peptide genes, *Drosomycin* (*Drs*), *Metchnikowin* (*Mtk*), *Attacin* (*Att*) and *Diptericin* (*Dpt*), in *D. melanogaster* larvae injected with PBS (PBS), *A. japonica* venom (V), the venom with the lateral oviduct components (V+O), and control non-treated larvae (NT). Expression level of each gene was normalized with that of *rp49* in each sample. Data are given as means±SE for three separate measurements using 5 larvae each. In each gene, no significant difference was detected between PBS and V, or between PBS and V+O injected larvae ($P > 0.05$).

Figure 3. Effects of the venom and lateral oviduct components on *D. melanogaster* hemocytes. (A) Images of *D. melanogaster* hemocytes exposed to medium, *A. japonica*

venom, the venom with the lateral oviduct components, and the lateral oviduct components alone. EtBr stained dead hemocytes appear red (arrowhead), while hemocytes engulfed FITC-labeled *E. coli* appear green (arrow). (B) Survival rates of hemocytes in each group. Data are given as means±SE for six separate measurements using 10 larvae each. Significant difference was detected between treatments (*P < 0.05).

Figure 4. Effects of the venom and lateral oviduct components on cellular immune responses of *D. melanogaster* hemocytes. (A) Percentages of spread hemocytes 30 min after treatment with medium (Medium), *A. japonica* venom components (V), the venom with the lateral oviduct components (V+O). Data are given as means±SE for four separate measurements using 10 larvae each. Significant difference was detected between treatments (*P < 0.05). (B) Percentages of hemocytes engulfed *E. coli* in each group. Other explanations are as in (A).

Figure 5. Protease-like activities in the plasma and hemocyte lysate of *Drosophila* larvae. (A) Protease-like activities in the plasma of *D. melanogaster* larvae at 4 hours post injection with PBS (PBS) and *A. japonica* venom components (V). Protease-like activities were measured by using synthetic peptide-MCA substrates that give fluorescence after the hydrolytic reaction. Data are given as means±SE for four separate measurements. Significant difference was detected between treatments (*P < 0.05). (B) Effect of time after injection of PBS (PBS), *A. japonica* venom components (V), or the venom with the lateral oviduct components (V+O) on the plasma protease-like (Boc-Ile-Glu-Gly-Arg-MCA hydrolyzing) activities in *D. melanogaster* larvae. Data are given as means±SE for eight separate measurements. Other explanations are as in (A). (C) Protease-like activities in the hemocyte lysate of *D. melanogaster* larvae using same substrates in (A). Other explanations are as in (A). (D) Effect of PBS (PBS), venom (V), or the venom with the lateral oviduct components (V+O) on the serine protease-like (Boc-Ile-Glu-Gly-Arg-MCA hydrolyzing) activities in the plasma of *D. ficusphila* larvae at 4 hours post injection of each sample. Other explanations are as in (B).

Figure 6. Comparison of toxicities of *A. japonica* and *A. rossica* venom components. (A) Percentages of *D. ananassae* and *D. auraria* larvae that died at the larval stage,

pupal stage, survived until eclosion, and produced adult wasp after uninterrupted (U) or interrupted (I) parasitism by *A. japonica*. (B) Percentages of *D. melanogaster* and *D. auraria* larvae that died at the larval stage, pupal stage, survived until eclosion, and produced adult wasp after uninterrupted (U) or interrupted (I) parasitism by *A.rossica*. Other explanations are as in (A). (C) Effects of PBS (PBS), *A. japonica* venom (V), or the venom with the lateral oviduct components (V+O) on survival rates of *D. ananassae* and *D. auraria* larvae by injection of each sample. Data are given as means±SE for three separate measurements using 3 larvae each. Significant difference was detected between treatments (*$P < 0.05$). (D) Effects of PBS (PBS) and *A. rossica* venom (V) on survival rates of *D. melanogaster* and *D. auraria* larvae by the injection. Difference between groups was not significant ($P > 0.05$).

Fig. 1

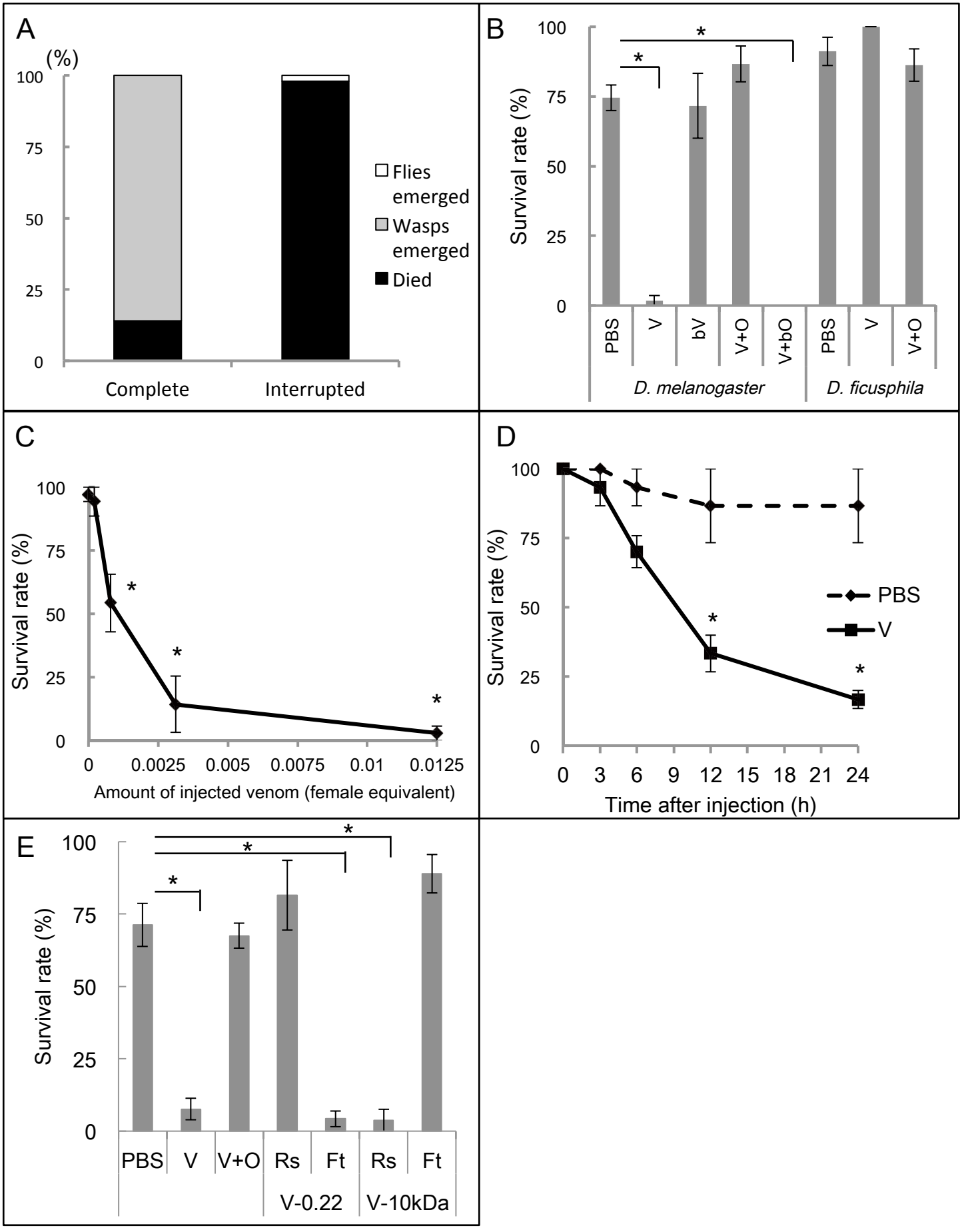

A
(%)
100
75
50
25
0
Flies emerged
Wasps emerged
Died
Complete
Interrupted
B
Survival rate (%)
PBS
V
bV
V+O
V+bO
PBS
V
V+O
D. melanogaster
D. ficusphila
C
Survival rate (%)
Amount of injected venom (female equivalent)
0
0.0025
0.005
0.0075
0.01
0.0125
D
Survival rate (%)
PBS
V
Time after injection (h)
0 3 6 9 12 15 18 21 24
E
Survival rate (%)
PBS
V
V+O
Rs
Ft
Rs
Ft
V-0.22
V-10kDa

Fig. 2

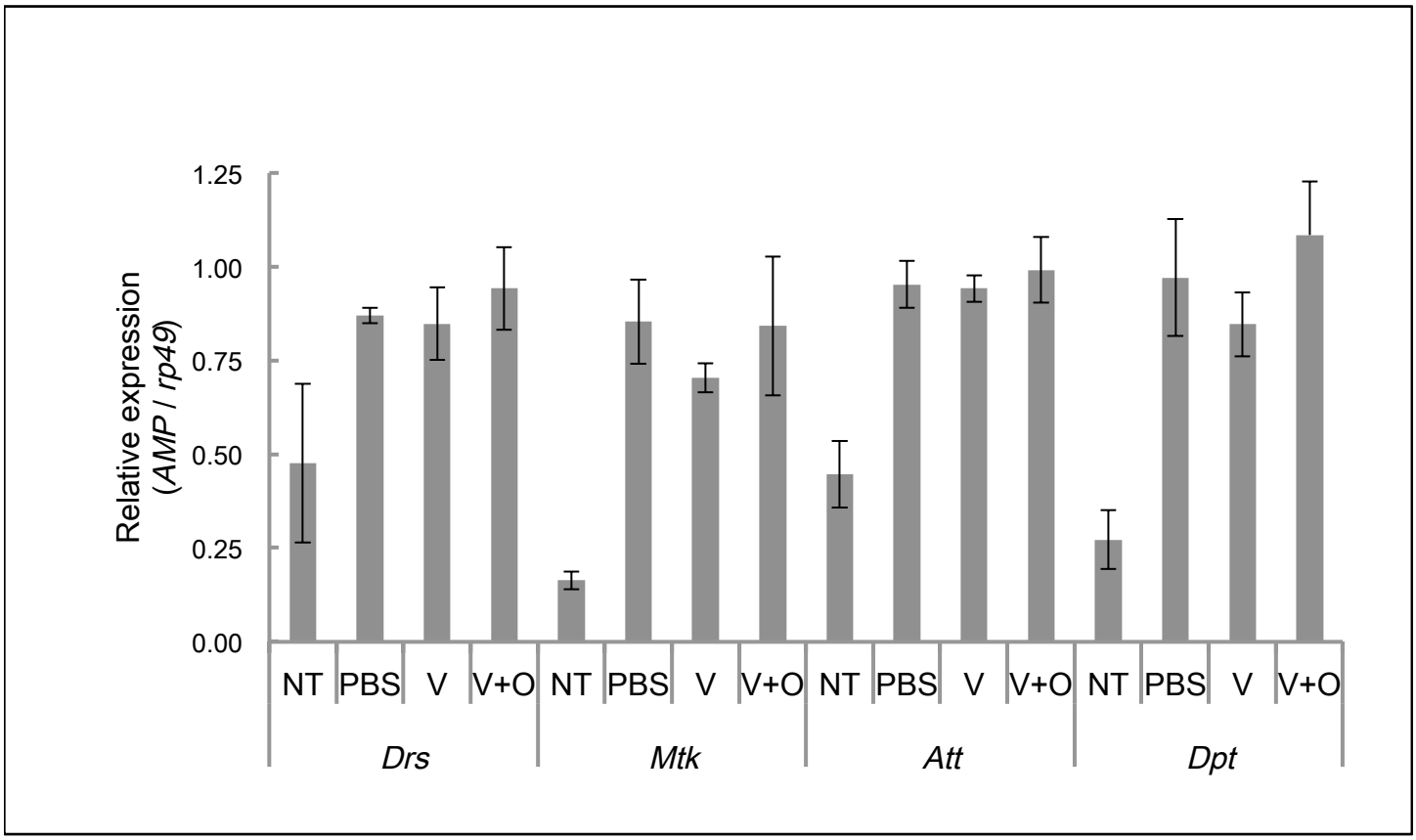
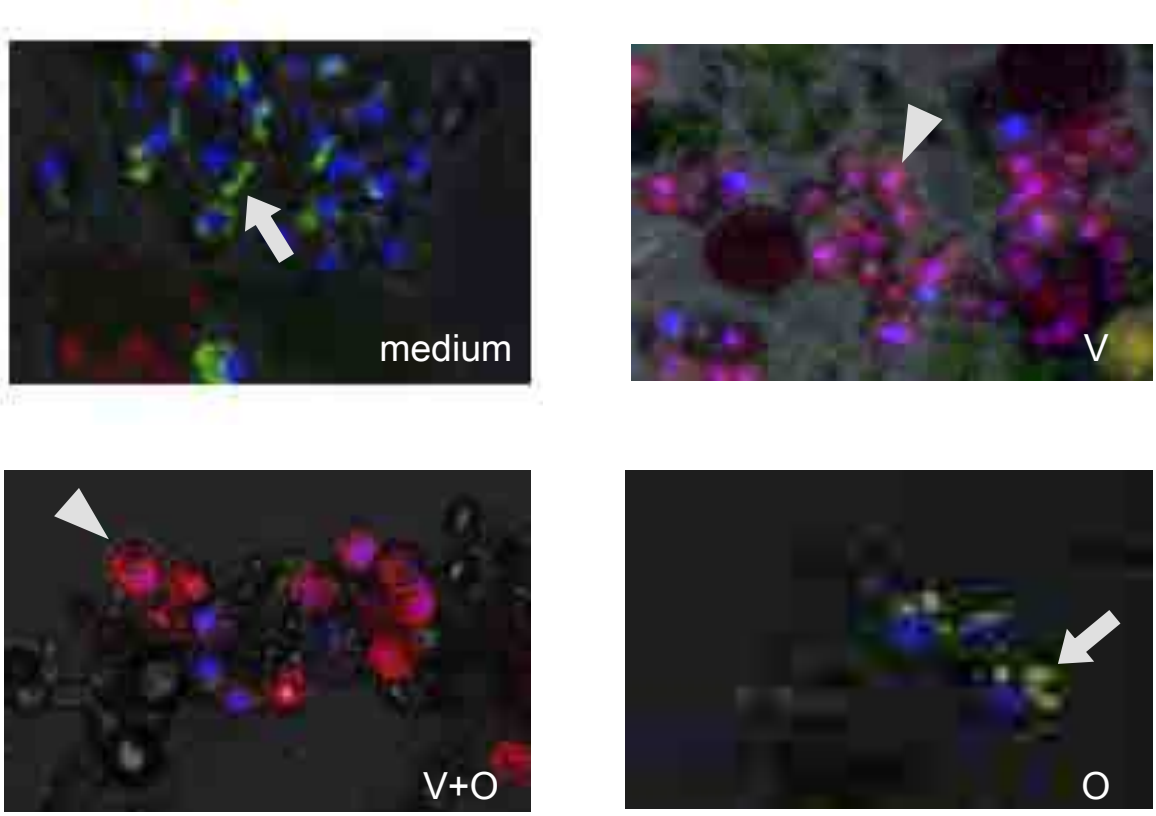

Relative expression
(AMP / rp49)
1.25
1.00
0.75
0.50
0.25
0.00
NT
PBS
V
V+O
Drs
Mtk
Att
Dpt

Fig. 3

A



B

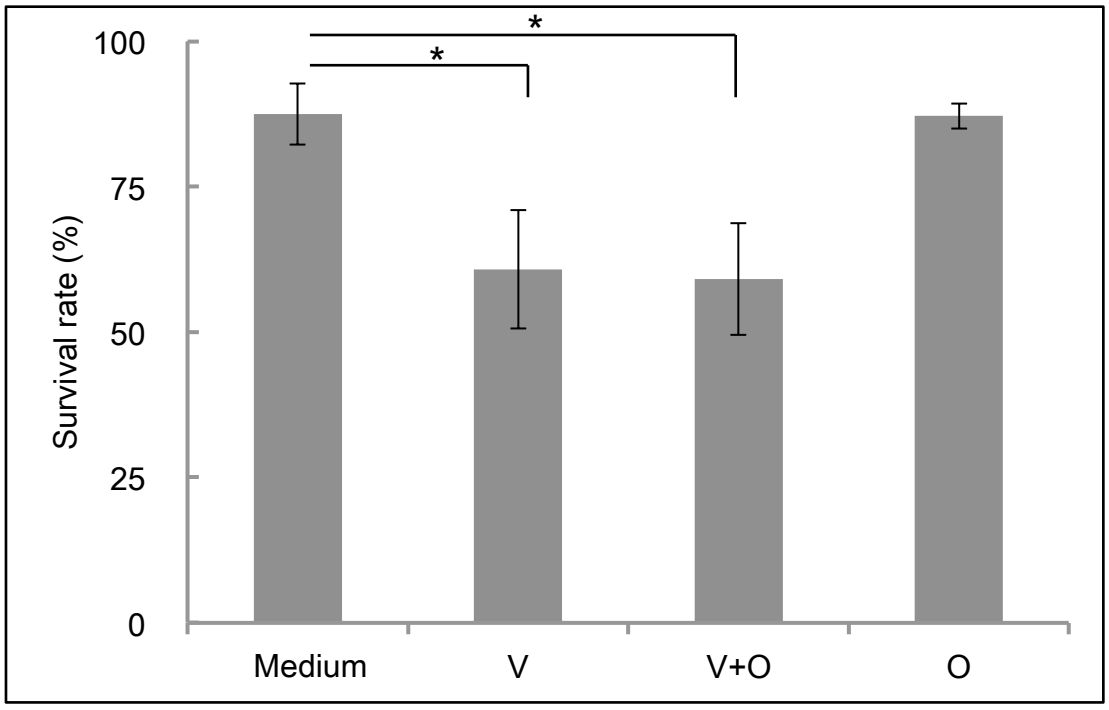


Fig. 4

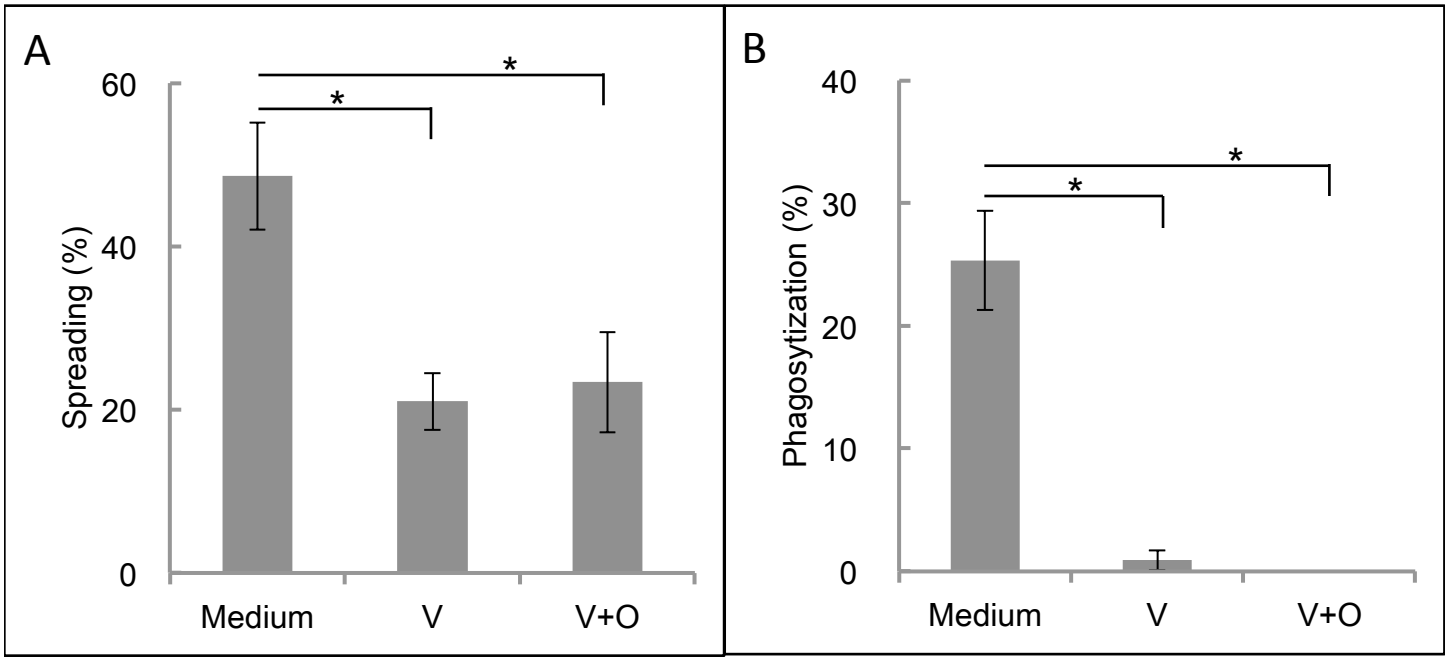

A
60
40
20
0
Spreading (%)
*
*
Medium
V
V+O
B
40
30
20
10
0
Phagosytization (%)
*
*
Medium
V
V+O

Fig. 5

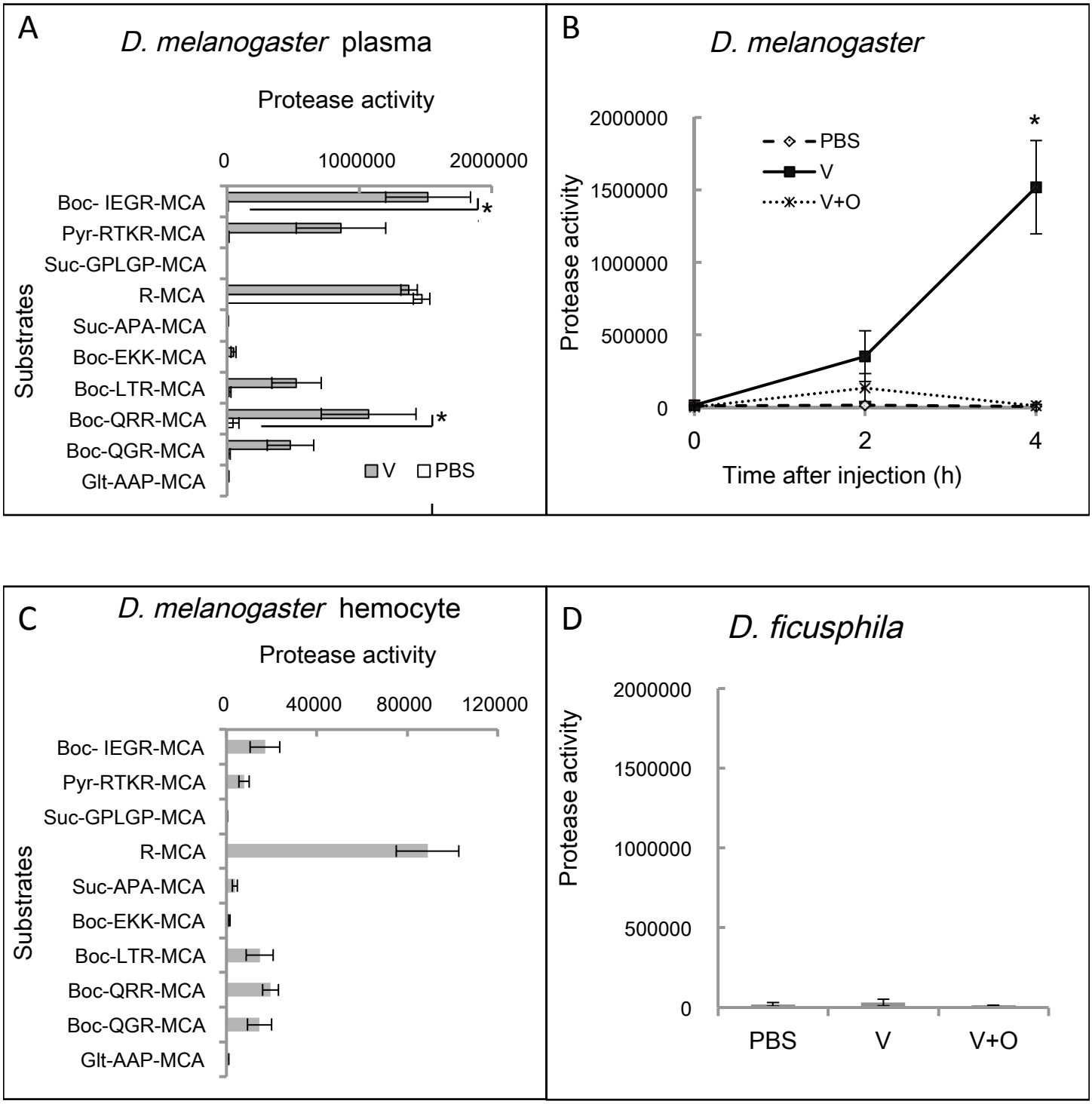

A
D. melanogaster plasma
Protease activity
0
1000000
2000000
Boc- IEGR-MCA
Pyr-RTKR-MCA
Suc-GPLGP-MCA
R-MCA
Suc-APA-MCA
Boc-EKK-MCA
Boc-LTR-MCA
Boc-QRR-MCA
Boc-QGR-MCA
Glt-AAP-MCA
Substrates
V
PBS
*
*
B
D. melanogaster
Protease activity
2000000
1500000
1000000
500000
0
PBS
V
V+O
*
0
2
4
Time after injection (h)
C
D. melanogaster hemocyte
Protease activity
0
40000
80000
120000
Boc- IEGR-MCA
Pyr-RTKR-MCA
Suc-GPLGP-MCA
R-MCA
Suc-APA-MCA
Boc-EKK-MCA
Boc-LTR-MCA
Boc-QRR-MCA
Boc-QGR-MCA
Glt-AAP-MCA
Substrates
D
D. ficusphila
Protease activity
2000000
1500000
1000000
500000
0
PBS
V
V+O

Fig. 6

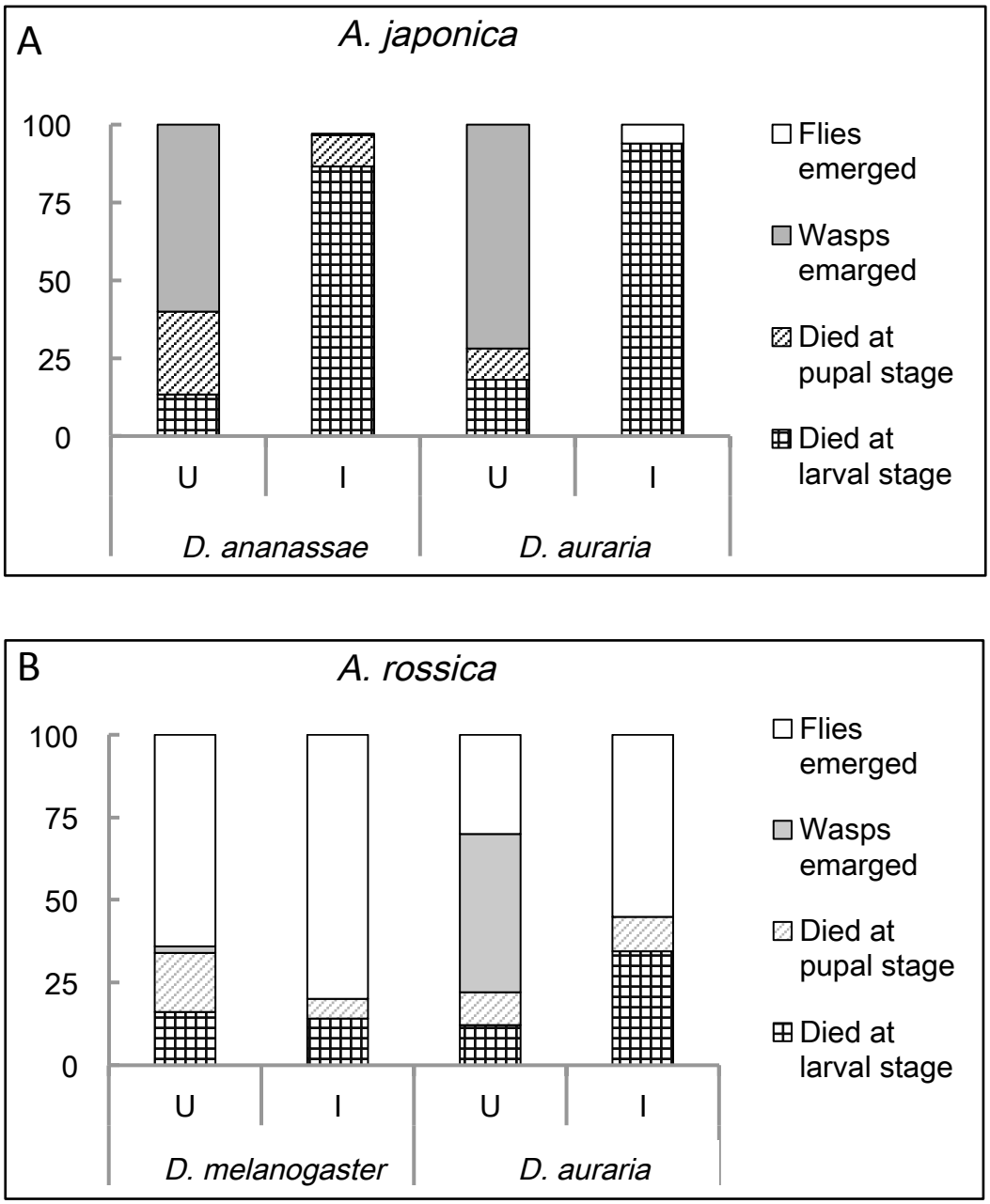
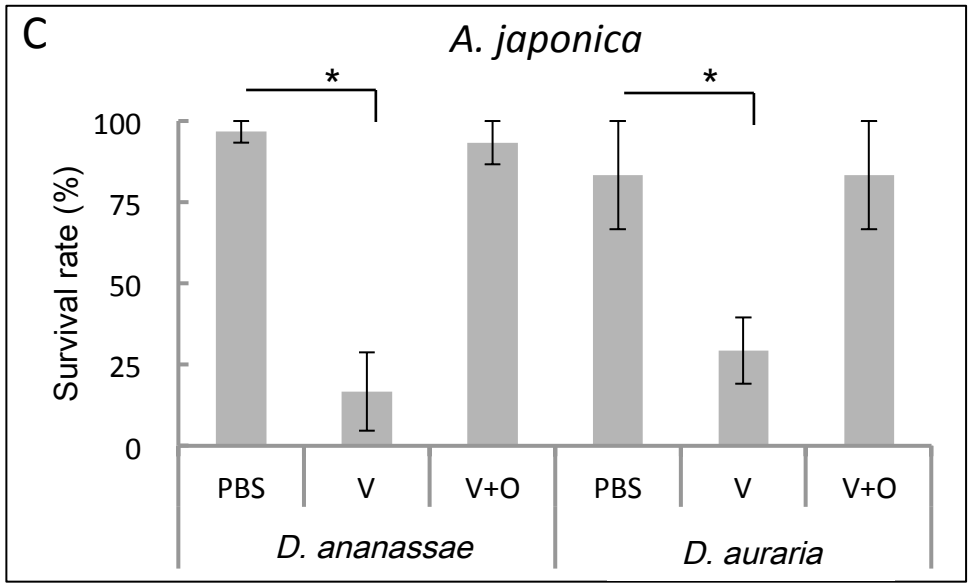
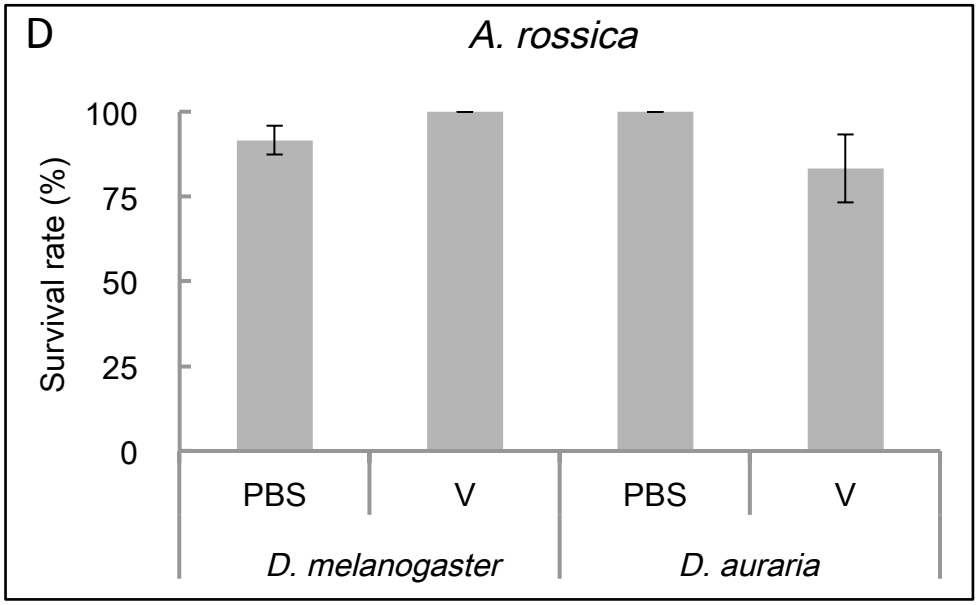

A
A. japonica
100
75
50
25
0
U
I
U
I
D. ananassae
D. auraria
Flies emerged
Wasps emarged
Died at pupal stage
Died at larval stage
B
A. rossica
100
75
50
25
0
U
I
U
I
D. melanogaster
D. auraria
Flies emerged
Wasps emarged
Died at pupal stage
Died at larval stage

Fig. 6


C
A. japonica
*
*
100
75
50
25
0
Survival rate (%)
PBS
V
V+O
PBS
V
V+O
D. ananassae
D. auraria


D
A. rossica
100
75
50
25
0
Survival rate (%)
PBS
V
PBS
V
D. melanogaster
D. auraria