Toxicity of venom of *Asobara* and *Leptopilina* species to *Drosophila* species

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Running title: *Effects of venom of* Asobara *and* Leptopilina

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Abstract. The *Drosophila* parasitoid *Asobara japonica* has highly toxic venom that kills host larvae if its injection is not followed by injection of lateral oviduct components along with egg-laying. In this study, the venom of seven other *Drosophila* parasitoids (*Asobara rossica, A. rufescens, A. pleuralis, Leptopilina heterotoma, L. japonica, L. ryukyuensis,* and *L. victoriae*) are tested against three kinds of *Drosophila* species (i.e. *Drosophila* species that are suitable as host for focal parasitoids, those that are resistant to the parasitoids, and a cosmopolitan species, *D. simulans*). Venom of the three *Asobara* species are not toxic to any of *Drosophila* species, whereas those of the four *Leptopilina* species are toxic to some *Drosophila* species. Toxicity of venom vary among *Leptopilina* species, and susceptibility to venom also vary among host *Drosophila* species. Furthermore, toxicity and paralytic effects of venom are not correlated. Because toxicity of venom is not adaptive for parasitoids, it may be an inevitable side effect of some components that play an essential role in parasitism.

Key words. *Asobara*, *Drosophila*, *Leptopilina*, parasitism, toxicity, venom.

Introduction

In oviposition, parasitoid females not only lay eggs in hosts but also inject them with venom that are complex mixtures of proteins and low molecular weight compounds having various functions such as induction of paralysis or regulation of host development and immune responses. Some components of venom act alone, while some act in combination with other factors provided from the mother's organs, parasitoid embryo or teratocytes (Moreau & Guillot 2005; Asgari & Rivers, 2011; Poirié *et al*., 2014). An interesting example of cooperative action of venom and oviduct products has been reported for *Asobara japonica* Belokobylskij, a parasitoid of *Drosophila* species. During parasitization of *Drosophila* hosts, *A. japonica* females inject venom into the host immediately after the insertion of the ovipositor (Furihata & Kimura, 2009). A few seconds later, parasitoid females vibrate their ovipositors for a few seconds and then withdraw them. Thus, vibration of the ovipositor is an indication of egg laying (Vet & Bakker, 1985; van Lenteren *et al.*, 1998; Dubuffet *et al*., 2006). In parasitism experiments using *A. japonica* and some host species such as *Drosophila melanogaster* Meigen and *D. simulans* Sturtevant, interruption of oviposition after injection of venom but before egg laying kills host larvae (Furihata & Kimura, 2009; Mabiala-Moundoungou *et al*., 2010; Furihata *et al*., 2013). However, this toxic action of the venom is suppressed by lateral oviduct components that are injected to the host by the parasitoid female along with egg (Mabiala-Moundoungou *et al*., 2010; Furihata *et al*. 2013). Additional studies showed that the venom of *A. japonica* is much less toxic to certain *Drosophila* species that kill *A. japonica* embryos or larvae at high rates, such as *D. bipectinata* Duda and *D. ficusphila* Kikkawa & Peng (Furihata & Kimura 2009; Furihata *et al.*, 2013). These "resistant" *Drosophila* species may have evolved some

mechanisms to detoxify lethal components of *A. japonica* venom along with the acquisition of capacity to kill *A. japonica* embryos and/or larvae in the course of their coevolutionary interactions. On the other end of the spectrum is *A. rossica* Belokobylskij, whose venom is not known to be toxic to any *Drosopila* species (Furihata *et al.*, 2013). Thus, effects and functions of venom vary among different parasitoid species. However, the toxicity of venom of several other *Drosophila* parasitoids has not been investigated (Moreau & Guillot 2005; Asgari & Rivers, 2011; Poirié *et al*. 2014).

In this paper, toxicity and paralytic effects of venoms of seven *Drosophila* parasitoids including three *Asobara* (Braconidae) species (*A. rossica*, *A. rufescens* (Förster) and *A. pleuralis* (Ashmead),) and four *Leptopilina* (Figitidae) species (*L. heterotoma* (Thompson), *L. japonica* Novković & Kimura, *L. ryukyuensis* Novković & Kimura and *L. victoriae* Nordlander) on three kinds of *Drosophila* species (suitable host, resistant species and *D. simulans*) are investigated to understand how the function of venom has evolved. *A. rossica*, *A. rufescens, L. heterotoma* and *L. japonica* are mainly distributed in the temperate regions of Asia and/or Europe, whereas the other three are distributed in subtropical and tropical regions of Asia and/or Africa (Allemand *et al*. 2002; Novković *et al*. 2011; Nomano *et al*. 2015). They show species-specific host use, and therefore some *Drosophila* species are suitable as host for some parasitoid species but some are resistant (Novković *et al*. 2012; Kimura & Suwito 2012, 2014; Kimura & Novković 2015; Kimura unpublished data). Little is known about the virulence strategy of these parasitoids, except for *L. heterotoma* and *L victoriae* that show active suppression of the host immune systems (Rizki & Rizki 1984; Morales *et al.*, 2005).

Materials and methods

Laboratory strains

Laboratory strains of *A. rossica*, *A. rufescens* and *L. heterotoma* were collected from Sapporo (43.0 °N, 141.2 °E), northern Japan in August 2012, that of *L. japonica* from Tokyo (35.7 °N, 139.8 °E), central Japan in June 2010, that of *L. ryukyuensis* from Iriomote-jima (24.2 °N, 123.8 °E) in March 2006, southern Japan, and those of *A. pleuralis* and *L. victoriae* from Kota-Kinabalu (5.3 °N, 117.4 °E), Malaysia in March 2008 (Table 1). Clumps of banana were placed in the field to allow drosophilid flies to oviposit in banana and parasitoid individuals to oviposit in drosophilid larvae in banana. Usually after a week, the bananas were brought back to the laboratory and placed in plastic containers. When drosophilid pupae were formed, they were collected in plastic Petri dishes, and examined for emergence of flies or parasitoids. When parasitoid individuals emerged, they were reared on appropriate hosts.

At the same time of parasitoid collection, a number of *Drosophila* species were collected from each locality. Suitability of these *Drosophila* species as host for sympatric populations of parasitoids was examined in our previous studies (Novković *et al*. 2012; Kimura & Suwito 2012, 2014; Kimura & Novković 2015; Kimura unpublished data). From *Drosophila* species collected in each locality, two species were chosen for the present experiments; one was a suitable host that allows focal parasitoid to develop successfully and another was a resistant species that kills embryos or larvae of focal parasitoid at high rates. In the experiments on *A. pleuralis* (from Kota Kinabalu), however, *D. parabipectinata* and *D. pseudoananassae* strains that were collected from Deramakot located approximately 200 km east of Kota Kinabalu in

March 2005 were used. In addition to *Drosophila* strains from the same or nearby localities, a *D. simulans* strain collected from Tokyo in June 2007 was used.

These parasitoid and *Drosophila* strains originated from few to several field collected females. The *Drosophila* strains were maintained with Drosophila medium. For the maintenance of parasitoid strains, *D. simulans* was used as host with the exception of *A. rossica* and *A. rufescens* strains which were maintained on *D. auraria* Peng (because *D. simulans* was highly resistant to these parasitoids). Rearing and experiments were performed at 23 °C under an LD 15:9 h photocycle, and experiments.

Parasitism experiments

Female parasitoids used in the experiments were maintained for two to five days in vials containing Drosophila medium with host larvae after emergence, i.e., they were fed, mated and had experience of oviposition. *Drosophila* larvae used in the experiments were 2-3 day old (mostly second-instar). Parasitoid females were placed with *Drosophila* larvae in Petri dishes (3 cm in diameter) with small amounts of food medium, and were observed for oviposition behaviour under a stereoscopic microscope. To obtain host larvae in which venom was injected but egg was not laid, host larvae were drawn apart from parasitoid females using forceps before the females started to vibrate their ovipositors (‘interrupted’ group). In another group, parasitoids were allowed to complete oviposition without interruption (‘un-interrupted’ group). Immediately after injection of venom or completion of oviposition, *Drosophila* larvae were transferred to new vials with food and examined for emergence of adult parasitoids or flies. Usually 50 *Drosophila* larvae were used for each treatment and more than five parasitoid females were used to obtain 50 treated-larvae.

In parasitism experiments, *Drosophila* larvae showed various degrees of paralysis, strong paralysis, weak paralysis and no sign of paralysis. However, it was not easy to determine the boundary between weak and no paralysis, because weakly paralyzed larvae were able to move more or less. On the other hand, strong paralysis was distinctive; i.e., larvae did not move at all even if stimulated by forceps. In addition, the occurrence of strong paralysis did not vary within strain; i.e. all larvae of a strain showed the same response. Thus, *Drosophila* larvae subjected to the above experiments were checked for the occurrence of strong paralysis when transferred from Petri dishes to new vials.

Statistical analysis

The frequency of individuals from which no insect (i.e., neither parasitoid nor fly) emerged was compared between the 'un-interrupted group' and 'interrupted group' by a generalized liner model (GLM) with binomial error distribution and logit-link function using R 3.2.1 (R Core Team 2015). The significance of the explanatory variable of the model was tested with likelihood ratio test.

Results

Figure 1 shows the results of the experiments using previously reported data for *A. japonica* (Furihara & Kimura, 2009) as a reference. For the *Asobara* species (*A. rossica, A. rufescens* and *A. pleuralis*), the frequency of individuals from which no insect (i.e., neither parasitoid nor fly) emerged did not exceed 40%, even if oviposition was interrupted before egg-laying. However, the frequency was significantly different

between the ‘interrupted’ and ‘un-interrupted’ groups for suitable hosts in the experiments on *A. rossica* and *A. rufescens* and for *D. simulans* in the experiment on *A. pleuralis* (GLM with likelihood ratio test, $P < 0.05$).

In *Leptopilina* species, the frequency of individuals from which no insect emerged was always higher in the ‘interrupted’ group than in the ‘un-interrupted group, and the difference was significant in all comparisons (GLM with likelihood ratio test, $P < 0.05$), except for *D. simulans* in the experiment on *L. japonica* and for suitable-host and resistant species in the experiment on *L. victoriae*.

Larvae of *D. simulans* always showed strong paralysis when parasitized by any of the seven parasitoids (data not shown). In addition, both suitable-host (*D. auraria*) and resistant species (*D. bifasciata*) showed strong paralysis when parasitized by *A. rossica* (Table 1). Furthermore, a resistant species (*D. biauraria*) showed strong paralysis when parasitized by *A. rufescens*, and a suitable-host species (*D. nigromaculata*) showed strong paralysis when parasitized by *L. heterotoma* (Table 1). The other parasitoids did not induce strong paralysis in suitable-host or resistant species. Thus, there was no clear relation between the occurrence of strong paralysis and toxicity of venom or suitability as host.

Discussion

Asobara japonica has venom that exhibits a toxic effect to host species if its injection is not followed by an injection of lateral oviduct components (Mabiala-Moundoungou *et al*., 2010; Furihata *et al*., 2013). However, the venom of the other three *Asobara* species studied here does not have such toxic effects. Four *Leptopilina* species have venom that exhibits toxicity to some *Drosophila* species if egg laying is not followed; i.e., *L.*

heterotoma venom is toxic to a suitable-host species, *L. japonica* venom to both suitable-host and resistant species, and *L. ryukyuensis* and *L. victoriae* venom to *D. simulans*. However, the frequency of *Drosophila* larvae from which neither flies nor parasitoids emerge is always higher when oviposition is interrupted. In this study, interruption of oviposition is achieved by separating *Drosophila* larvae from ovipositing parasitoid females by forceps. *Leptopilina* species have an ovipositor clip by which they hold host larvae during oviposition (van Lenteren *et al.*, 1998; Buffington, 2007), and *Drosophila* larvae are sometimes dragged by parasitoids when larvae and parasitoids are separated by forceps (Kohyama, personal observation). It is possible that the separation by forceps may injure *Drosophila* larvae and increase their mortality to some degree. In the above cases of *Leptopilina*, however, the frequency of *Drosophila* larvae from which no insect emerges is very high (> 84%), which suggests that their venom has at least some toxicity to some *Drosophila* species. For a further understanding of the toxicity of *Leptopilina* venom, artificial injection of venom is required.

It is not known why *A. japonica* and the four *Leptopilina* species have toxic venom. In koinobiont larval parasitoids, such as *Asobara* and *Leptopilina* species, the toxicity of the venom is not adaptive because parasitoids are required to allow host individuals to survive and develop to the pupal stage after parasitization. The toxicity of their venom may be an inevitable side effect of some components that play an essential role in the regulation of host development or suppression of host immune responses. In this study, the toxicity of venom varies among the parasitoid species, and each parasitoid's venom shows different toxicity to different *Drosophila* species. Toxicity is not related to the occurrence of strong paralysis. These results suggest that the parasitoid's systems to regulate host development and immune responses are complicated and have species-specific components (Dupas *et al*., 2003, 2009;

Thompson, 2005; Kimura & Suwito, 2014). Biochemical and molecular biological studies would provide some insight into how the virulence mechanisms of the toxicity of venom has evolved and diversified.

Drosophila simulans is strongly paralysed by venom of all of the parasitoid species studied. *Drosophila simulans* is an invasive species originating in Africa (Lemeunier *et al.*, 1986); therefore, it has a relatively short history of interaction with the Palearctic and Asian parasitoid species studied here and may not have had sufficient time to adapt to these parasitoid species. In this respect, it is interesting how *D. simulans* responds to parasitoids of the original locality of Africa.

In the experiments with suitable-host and resistant *Drosophila* species, strong paralysis is observed only in one case (i.e., *D. nigromaculata* oviposited by *L. heterotoma*) out of eight cases in which *Leptopilina* species were used, whereas in five cases out of eight cases in which *Asobara* species were used (including *A. japonica*: Furihata & Kimura 2009). Thus, *Asobara* venom induces strong paralysis in a larger number of *Drosophila* species. This characteristic may be related to the absence of the clip-on ovipositor in *Asobara* species. In our observation (Kohyama, personal observation), the third instar larvae of *D. auraria* can easily escape from the attack by *A. rufescens* because *A. rufescens* does not have an ovipositor clip nor the capacity to paralyse *D. auraria* larvae. Therefore, many *Asobara* species may have intensified the paralytic effects of venom in compensation to the absence of an ovipositor clip.

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Figure Legends

Fig. 1. Percentages of fly larvae from which flies (white), parasitoids (grey) or neither organism (black) emerged when *D. simulans*, suitable-host and resistant species were parasitized by *Asobara* (A–D) and *Leptopilina* species (E–H) or injected with their venom (I: oviposition of parasitoid females was interrupted after venom injection but before egg-laying, U: oviposition was not interrupted). Suitable-host and resistant species for each parasitoid species is given in Table 1. Numbers above bars indicate the number of individuals used in each experiment, and symbols above bars indicate results of statistical tests between 'interrupted' (I) and 'un-interrupted' (U) groups (GLM with likelihood ratio test, $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$). Data of *A. japonica* were from Furihata & Kimura (2009)

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Table 1. Parasitoid and *Drosophila* species used in the experiments, and the original localities of the experimental strains. Species that showed strong paralysis when parasitized by parasitoids given in the left column was shown in bold.

	Susceptible host species	Resistant species	Original locality
*A. japonic*a*	***D. auraria* Peng***	***D. bipectinata* Duda****	Tokyo, Iriomote-jima
A. rossica	***D. auraria***	***D. bifasciata* Pomini**	Sapporo
A. rufescens	*D. auraria*	***D. biauraria* Bock & Wheeler**	Sapporo
A. pleuralis	*D. parabipectinata* Bock	*D. pseudoananassae* Bock	Kota Kinabalu
L. heterotoma	***D. nigromaculata* Kikkawa & Peng**	*D. auraria*	Sapporo
L. japonica	*D. rufa* Kikkawa & Peng	*D. auraria*	Tokyo
L. ryukyuensis	*D. ananassae* Doleschall	*D. bipectinata*	Iriomote-jima
L. victoriae	*D. malekotliana* Parshad & Paika	*D. bipectinata*	Kota Kinabalu

*From Tokyo,**from Iriomote-jima (Furihata and Kimura 2009).

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