

CHAPTER 2. VENOMOUS SALIVA: OPTIMIZATION AND UTILIZATION

2.1. ABSTRACT

Milking and electrical stimulation methods were used for the venomous saliva (VS) collection by subjecting the *Rhynocoris fuscipes* (Fab.) to continuous (CMM) (1, 2, 3, 4, 5 and 6 days) and discontinuous (DMM) (1, 3, 5 and 7 days) food deprivation levels. The quantity of VS production and utilization were examined by providing *Corcyra cephalonica* (Stainton), *Spodoptera litura* (Fab.) and *Helicoverpa armigera* (Hubner). The male predators significantly secreted more VS than the females both in the continuous and discontinuous prey deprivation of both manual milking and electrical stimulation methods. The 100% survival (SR) and venom milking rate (VMR) were observed in continuous milking method. In discontinuous manual milking (DMM), 20% each SR and VMR were recorded and similarly in the continuous electrical stimulation (CES) 30% SR and 20% VMR and in discontinuous electrical stimulation (DES), 20% SR and 30% VMR were recorded. The quantity of protein was high in the one day starved female VS milked in the DES. *Spodoptera litura* fed *R. fuscipes* secreted higher quantity of VS than *H. armigera* and *C. cephalonica* fed groups. The predator uses the maximum quantity of VS to paralyze the *S. litura* than the *C. cephalonica* and *H. armigera*. For the maximum VS yield, continuous manual milking method fed with *S. litura* can be used for the VS optimization and utilization.

2.2. INTRODUCTION

The heteropteran predators do not confine them to the body yields of their prey, as is so after or implied (Cohen, 1996). Instead, they use a solid to liquid feeding method by attaching the nutrient rich solid or semi-solid organs and tissues of prey (Cohen and Patana, 1984, 1985; Cohen, 1998a; Sahayaraj *et al.*, 2010). Venomous insects are known from the orders Lepidoptera (Bene *et al.*, 1999); Hemiptera (Maran, 1999; Ambrose and Maran, 1999b; Maran and Ambrose, 2000; Sahayaraj *et al.*, 2006 a, b; 2010; Sahayaraj and Vinoth Kanna, 2009) and Hymenoptera (Blum, 1981; Ućkan *et al.*, 2004; Rivers *et al.*, 2006). Venom consists of a complex mixture of toxic components that include protein and peptide toxins, enzymes and other active biomoleclues (Corzo *et al.*, 2001; Sahayaraj *et al.*, 2010; Sahayaraj and Muthukumar, 2011). These molecules serve the dual purposes of prey paralysis and digestion and / or defense against other predators (Bailey and Wilce, 2001;

Palma 2006; Rivers *et al.*, 2006; Sahayaraj *et al.*, 2006a; Turillazzi *et al.*, 2006; Sahayaraj and Vinoth Kanna, 2009; Sahayaraj *et al.*, 2010).

The venom milking is used for the collection of venom and preventing the venom contamination by non-venom enzymes (Rash and Hodgson, 2002). Three different methods have been used for the VS/true venom collection: a) manual milking method in reduviids (Sahayaraj *et al.*, 2006 a, b; Sahayaraj and Vinoth Kanna, 2009; Kumar, 2011); in hymenoptera (Piek, 1986; Funari *et al.*, 2001; Deyrup and Matthews, 2003; Hisada *et al.*, 2005) and in spiders (Vonarx *et al.*, 2006); b) electric stimulation methods in reduviidae (Barbosa *et al.*, 1999; Corzo *et al.*, 2001; Sahayaraj *et al.*, 2006a; Kumar, 2011); in hymenoptera (Funari *et al.*, 2001); in spiders (Johnson *et al.*, 1998; Herzig and Hodgson, 2008; Herzig *et al.*, 2008; Rocha-e-Silva *et al.*, 2009); in scorpions (Possani *et al.*, 2000; Incesu *et al.*, 2007) and C) whole gland extraction in reduviids (Maran, 1999; Ambrose and Maran, 1999; Maran and Ambrose 2000); ants (Haight and Tschinkel, 2003; Chen *et al.*, 2009); in wasps (Uçkan *et al.*, 2004; Rivers *et al.*, 2006); in spiders (Silva *et al.*, 2008).

Reduviids are mostly insectivorous predators which use their VS as a tool of aggression in order to paralyze or immobilize rather than kill their prey. Venomous saliva is synthesized in relatively small quantities (Maran, 1999) in the paired principal gland (Cohen, 2000; Sahayaraj *et al.*, 2010). Since reduviids capture several prey items per day (Ambrose, 1999; Sweet, 2000; Sahayaraj, 2007), one would expect, that they would strictly control the quantity of VS released according to prey type. Injection of too much VS into smaller preys could be metabolically expensive and may deplete venom reserves, leaving the reduviid vulnerable to predation or unable to deal with subsequent prey. No attempts have been made to quantify the amount of VS injected by reduviids into different prey items. It has been reported widely that reduviids take less time while they are in starvation (Maran, 1999, Ambrose and Maran, 1999; Maran and Ambrose, 1999) and provided with small preys. The gender of the animal also influences the quantity of VS released, the female predators secrete more quantity of VS by *R. marginatus* and *Catamirus brevipennis* (Sahayaraj *et al.*, 2006a) and a similar observation was made by Sahayaraj and Vinoth Kanna (2009) in *C. brevipennis* by discontinuous milking method. Very recently, Kumar (2011) took an attempt to quantify VS, when *Rhynocoris marginatus* (Fab.) subjected to continuous (1, 2, 3, 4, 5, 6 and 7 days) and discontinuous (1, 3, 5, 7 and 9 days) prey deprivation. However, no one has made an attempt to quantify the VS secreted in relation to manual milking, electrical stimulation by

combining while the *R. fuscipes* (Fab.) was subjected to continuous (1, 2, 3, 4, 5 and 6 days) and discontinuous (1, 3, 5 and 7 days) prey deprivation period. The evolution of venomous and poisonous organisms, whether toxic compounds are acquired through the development of a venom producing organ or through the external sources (says Mebs, 2001) is essential. The serving of the main nerve supply to the venom glands does not affect venom production, protein concentration or enzyme activity. It has been suggested previously that venom production is not under nervous control (Kochva, 1978). Maran (1999) has studied total body carbohydrate, protein and lipid of *R. fuscipes*, *R. marginatus* and *Rhynocoris kumarii* and has fed different pest such as *Spodoptera litura* (Fab.), *Dysdercus cingulatus* (Fab.) and *Mylabris pustulata* (Thungberg).

The objectives of this study were: 1) to optimize the VS secretion by different methods (manual milking and electrical stimulation) while *R. fuscipes* was subjected to continuous and discontinuous prey deprivation periods; 2) to evaluate the impact of preys on VS milking on different pests (*S. litura*, *H. armigera* and *C. cephalonica*) and 3) to evaluate the utilization of VS by different prey.

2.3. MATERIALS AND METHODS

2.3.1. Insects Collection

The adult male and female *Rhynocoris fuscipes* used for the study were taken from our permanent breeding stock. The animals were kept under the following 30 ± 0.21 °C temperature, $71.68 \pm 0.41\%$ RH and a photoperiod of 13L: 11D h. The adults were kept individually in polystyrene boxes (5 x 4 cm) with *C. cephalonica*. All boxes have a layer of absorbent paper at the bottom. Also cleaning occurs weekly.

Life stages of armyworm, *Spodoptera litura* (Fab) and American bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) were collected from the castor, groundnut and bhendi agro-ecosystems of Tirunelveli District, Tamil Nadu. The pest life stages were reared using their natural host under the above mentioned laboratory conditions. The laboratory emerged F₁ or F₂ generation life stages were used for the studies.

2.3.2. Venomous saliva optimization

2.3.2.1. Starvation and collection method

The five-day old *R. fuscipes* male and female were subjected to continuous (1, 2, 3, 4, 5 and 6 days) (CPD) and discontinuous (1, 3, 5 and 7 days) (DPD) prey deprivations. For CPD category predators were fed *ad libitum* before the commencement of the experiment. After this, the animals of this group did not receive food for 6 days, whereas, DPD category, after 1, 3, 5 and 7 days of starvation, the predators were subjected to VS milking, then the predators received food in *ad libitum*. After each prey deprivation period, the VS were collected once in the glass capillary tube (6 cm long, 2 mm outer diameter, 1 mm inner diameter) using milking and electrical stimulation method (Sahayaraj *et al.*, 2006a; Sahayaraj and Vinoth Kanna, 2009). The weight of the predators and a capillary tube were recorded before and after collection by milking method. To milk VS, *R. fuscipes* was held between two fingers – thumb on the ventral side of the abdomen and second finger on the dorsal side. Care was taken not to give too much stress to the animal during handling. Then a glass capillary tube was inserted into the tip of the rostrum. By gently pressing with the fingers, the insect was stimulated to insert the rostrum deeper into the capillary tube and eject the VS from the salivary gland. During this act, the venom flowed from the tip of the rostrum as thin drop of VS into the capillary tube. The act of gently pressing the abdomen of the animal was made twice or thrice, with an interval of 5-10 seconds each. The collection of the VS stimulated additional venom flow, as a result of either the pull on the capillary tube into the rostrum up to the second segment or the contact of the finger on abdominal hairs. Generally a milking lasts for a minute.

In the electric stimulation method, instead of holding the animals with fingers, the animals were held with a forceps attached with the electric shock inducer (Mahalakshmi Electric and Co., Tamil Nadu). After holding the animal, electric stimulus of 20 mV was passed into the abdominal sternal region either between fourth and fifth segment or fifth and sixth segments. The stimulation was given two or three times during one milking. After the VS collection, the weight of the predator and capillary tube was reweighed for quantifying the amount of VS milked. The survival rate (SR) and venom milking rate (VMR) were calculated using the following formulae:

$$\text{SR (\%)} = \frac{\text{No. of animals survived after the VS collected}}{\text{Total no. of animals subjected to VS milking}} \times 100$$

$$\text{VMR (\%)} = \frac{\text{No. of animals milked VS}}{\text{Total no. of animals subjected to VS milking}} \times 100$$

2.3.2.2. Prey type

Newly emerged *R. fuscipes* males were continuously fed for a period of 40-50 days with third stadium of *S. litura* (133.0 ± 1.9 mg), *H. armigera* (173.3 ± 2.6 mg) and fifth stadium of *C. cephalonica* (29.0 ± 0.4 mg) individually. In each category, 15 males were maintained. Then the predators were allowed to starve for a period of 3-day and VS was collected by manual milking methods as mentioned in the above section. The predatory efficiency of *R. fuscipes* have also been studied on the above three pest; by using the three days starved predators (pre-weighed) were released into the Petridish (9 cm) and followed by the individual prey item. Now the no. of sites pinned and paralyzing time were recorded.

2.3.3. Venomous saliva utilization

We performed this study with 10-days old *R. fuscipes* from our permanent breeding stock. Three different prey types were chosen for the bioassays: third instar larvae of *S. litura*, *H. armigera* and fifth instar larvae of *C. cephalonica*. We used the same culture which had been starved for 3-day and pre-weighed. In the feeding experiment, pre-weighed predator was released into a glass Petridish (9 cm diameter) (Borosil, India), then the pre weighed preys (2 each) were released. Now allow the predator to capture and inject the VS for exactly 5 min. Then both the predator and the prey were separated gently with a soft fine brush (Camalin, India) and weighed. The weight difference was considered as the amount of VS injected by a predator or the amount of VS utilized by a prey.

2.3.4. Quantification of VS for protein

The VS obtained from the predators in the prey deprivation and the different preys offered were used. The total protein content of different categories of VS was quantified using Lowry *et al.* (1951) method. In protein quantification, the VS was mixed with the 5 ml of reagent C (99 ml of reagent A: 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of reagent B: 1% $\text{Cu}_2\text{SO}_4 \cdot 5\text{H}_2\text{O}$ in 1% potassium sodium tartarate). The mixture was incubated at room

temperature for 10 min, and then 0.5 ml of three fold diluted folin-ciocalteou's reagent was added and re-incubated at room temperature for 30 mins. The blue colour developed was read at 750 nm against a blank (replacing distilled water instead of VS) in a spectrophotometer (Elico, India). The values obtained were compared with the standard (Bovine Serum Albumin, 1 mg/1 ml).

2.3.5. Statistical analysis

The statistical comparison was made between different days of starvation in both continuous and discontinuous starvation; one way analysis of variance (ANOVA) and the post hoc Tukey's test were also performed. The Box plots were made for the protein quantity of the VS yield by the starvation. Line column on 2 axes were performed for the VS yield was fed by *R. fuscipes* on different prey items. Box plot were made for the VS utilization by different prey items was made by using SPSS statistical software package Ver. 11.5 (SPSS Inc., 2005) and Microsoft excel.

2.4. RESULTS

2.4.1. Venomous saliva optimization

In continuous starvation, 3-day starved male secreted more quantity of VS both in milking (2.61 ± 0.28 mg/100 mg of animal wet weight) ($df = 6, 3; F = 0.660; p > 0.05$) and in electric stimulation (1.32 ± 0.06 mg/100 mg of animal wet weight) ($df = 6, 3; F = 5.17; p > 0.05$) method respectively (Table 2.1). In the discontinuous starvation in the milking method, five-day starved male milked (4.07 ± 0.60 mg/100 mg of animal weight) ($df = 7, 2; F = 14.85; p > 0.05$) more quantity of VS, whereas in the electric stimulation method three-day starved male (3.17 ± 0.46 mg / 100 mg of animal weight) milked more VS ($df = 7, 2; F = 7.865; p > 0.05$) (Table 2.2).

2.4.2. Survival (SR) and venom milking rate (VMR)

In continuous starvation, 100% of the male and female survived up to the sixth day of starvation, whereas, the VMR was high during the third day of starvation and gradually decreased to 70% at day six of starvation (Figure 2.1. a, b). In the electrical stimulation method, the VMR was more on the 3rd day of starvation, and gradually decrease to sixth day (Figure 2.1. c, d). In the discontinuous starvation of milking method, VMR gradually

increased from day one to day five day and decreased for the remaining starvation period. However, the survival rate was high on the third day of starvation, whereas in the discontinuous electrical stimulation method, the survival rate gradually decreased from the first day to seventh day of starvation (figure 2.1. g, h).

2.4.3. Quantification of protein of VS yielded in the prey deprivation

The protein quantity of *R. fuscipes* female was insignificantly high when VS was milked by electric stimulation method (df = 1, 9; F = 0.017; p > 0.05) whether the predator was subjected to continuous (Figure 2.2) or discontinuous (df = 12, 3; F = 0.683; p > 0.05) (Figure 2.3) starvation. In general, VS of female has more protein content than male.

2.4.4. Influence of prey on VS yield

Spodoptera litura fed *R. fuscipes* insignificantly milked more VS (df = 1, 9; F = 0.239; p > 0.05) than the *C. cephalonica* (1.15 ± 0.11 mg) and *H. armigera* (df=1, 9; F = 0.001; p > 0.05) (Figure 2.4). The protein content of VS obtained from *C. cephalonica* (48.80 ± 0.20 mg) was insignificantly higher than the *S. litura* (df = 1, 4; F = 0.028; p > 0.05) and *H. armigera* (df = 1, 4; F = 0.041; p > 0.05) (figure 2.4). From the predatory potential studies, it was found out that *R. fuscipes* took 32.38 ± 1.96 min to paralyze *H. armigera* and 8.00 ± 0.57 times pinned the prey. It was significantly higher than *S. litura* (16.19 ± 2.43 min; 5.20 ± 0.81) (df = 1, 18; F = 26.89; p < 0.05) and *C. cephalonica* (3.62 ± 0.43 mins and 3.10 ± 0.31) (df = 1, 18; F = 205.60; p < 0.05).

2.4.5. Utilization of VS by the prey

The quantity of VS injected into the three different prey types is shown in Figure 2.5. All prey were accepted prey types (100% attacked and paralyzed, n = 10). *Spodoptera litura* third instar larvae significantly received more quantity of VS (1.36 ± 0.53 mg) (df = 7, 2; F=1.175; p < 0.05) than the *C. cephalonica* (1.05 ± 0.22 mg) and *H. armigera* (0.59 ± 0.20 mg) (df=7, 2; F=2.130; p < 0.05).

2.5. Discussion

Reduviid venomous saliva has insecticidal (Corzo *et al.*, 2001; Kumar, 2011; Sahayaraj and Muthukumar, 2011), antimicrobial (Sahayaraj *et al.*, 2006b) and cytotoxic (Sahayaraj and Muthukumar, 2011) activities. However, major difficulty is in obtaining

sufficient amounts of high quality VS. Two methods have been used to obtain reduviid venomous saliva: salivary gland isolation and homogenization (Maran, 1999; Ambrose and Maran, 1999; Maran and Ambrose, 1999); manual milking method and electrical stimulation (Kumar, 2011; Sahayaraj and Muthukumar, 2011).

2.5.1. Prey deprivation

This is experimental evidence that spiders (Vapenik and Nentwig, 2000) and reduviid (Kumar, 2011; Sahayaraj and Muthukumar, 2011) are able to regulate the quantity of VS milked according to prey deprivation. In general, male secreted more VS than the female. This might be due to the factor that females utilize water from the salivary gland (reabsorption) for the body maintenance and fecundity (Miles, 1972; Miles and Slowiak, 1976). In the milking method, the male and the female showed 100% survival rate. The electric stimulation used to obtain VS from the reduviids through an electric stimulator was found to be stressful (such as: enhancing of VS release, leg contractions) or in extreme cases fatal. In the electric stimulation, the animal does not receive any markings due to the electric shock and while considering both milking and electrical stimulation, the milking method was found to be better which results in the high yield of VS and 100% animals survived up to 6 days of starvation. This was due to the limited stress given to the predator during the milking act. And also the third day of starved animals had high quantity of VS and it gradually decreased up to the sixth day of starvation. In contradiction, previously Ambrose and Maran (1999) and Maran and Ambrose (1999) demonstrated that the accumulation of VS in the salivary gland and increased starvation leads to loss in the size and quantity of VS and salivary gland. Size of the predator has not been considered in this study, because we converted all the value to 100 mg body weight of the predator.

Generally during starvation, predators do not use their VS and this leads to the accumulation of VS in the salivary gland. This indicates that the maximum quantity of VS is secreted by the predator in the third day of the starvation. Reabsorption of water makes the predator to live and this is provided during the sixth day of starvation with a very lower quantity of VS (0.02 ± 0.01 mg/100 mg of the animal weight). The third day of starvation was found to be an optimum period for the VS collection whereas previously Sahayaraj *et al.* (2006a) had shown that the female predators' *R. marginatus* and *Catamirus brevipennis* yield more VS than the male. Vapenik and Nentwig (2000) observed that in *Cupenis salei*, the venom quantity was decreased as the starvation period was increased. In our studies, the

quantity of VS yield by the male was high than that of the female, whereas in the *Bothrops insularis* females yield more quantity of venom than the male (Rocha-e-Silva *et al.*, 2009).

Protein play an important role in VS toxicity and the role assumes differential importance in various species of venoms animals. And in general, the cost of venom production may increase dramatically in predators with high feeding frequencies (Secor and Diamond, 2000). When compared to manual milking method, the electrical stimulation method was a painful procedure gives more stress to secrete the VS and so the animals secrete a more quantity of VS (Rocha-e-Silva *et al.*, 2009). Previously, Miles (1972) reported that the reabsorbing of the water from the salivary gland during the prey deprivation period makes the animals to live for a few more days. But in the electrical stimulation, the stimulus causes an irritant to the animal which may stress the animal to secrete the total quantity of VS which may lead to earlier depletion of VS and leads to the shrinking of the salivary gland (Ambrose and Maran, 1999). This some times may lead to the death of the animals. The electric stimulation of an *R. fuscipes* causes a recoverable paralysis which does not allow them to feed (Rocha-e-Silva *et al.*, 2009). The prevention of VS contamination by non-venom enzymes is an important consideration during VS collection (see Rash and Hodgson, 2002).

The protein quantity of the VS of female obtained from the electric stimulation method was found to be high. The animals were motivated to produce more quantity along with the salivary gland secretions and thus the level of protein quantity was high in the electric stimulation. The sex related divergence in the venom yields was quite small in the *Atrax* species (Atkinson and Walker, 1985) and *Cupenis salei* (Kuhn-Nentwig *et al.*, 2004).

2.5.2. Prey type and VS yield

The *S. litura* fed *R. fuscipes* secrete more quantity of VS than the other two pest. This might be due to the high nutrient (Figure 2.4) and size of the prey. Previously, Maran (1999) demonstrated that the *S. litura* fed predators' body carbohydrate, protein and lipid were higher. Recently, Kumar (2011) has shown that the soft bodied prey, *S. litura* was more preferred than the hard cuticle prey from Coleoptera and Hemiptera.

2.5.3. Venomous saliva utilization

From a reduviid's point of view *S. litura* is unproblematic prey type. They are not protected by thick chitinous exoskeleton and cannot escape by flight. However,

H. armigera try to escape by rolling and splitting behaviors (Sahayaraj, 1991), but the *R. fuscipes* attacked in the head region of the *H. armigera*. Moreover, due to the defensive nature of the *H. armigera* during the predation it attains a maximum time for its paralysis. Boeve *et al.*, (1995) described that the spiders inject more venom into a bigger than into a smaller prey. *Spodoptera litura* on contact, *R. fuscipes* showed a greater response, by releasing more quantity of defensive fluid from its mouth to defend the predators and this prompts the predators to provide more quantity of VS injected.

Perret (1977) reported that the spiders inject more quantity of venom into the larger prey (Boeve *et al.*, 1995) and in scorpions the same was recorded by Bub and Bowerman (1979); Cushing and Matharne (1980); Casper (1985); Rein (1993). The potentially high costs of venom production are frequently assumed to have evolutionary, ecological and behavioral consequences, to quantify the actual energetic costs of venom production (Pintor *et al.*, 2010). The quantity of VS differs depending upon the place of injection. If it injects near the nervous system it requires a low quantity of venom/Vs whereas the injection of VS into the abdomen by the predator requires more quantity (Kuhn Nentwig *et al.*, 1994, 2000). Maran (1999) has observed the nutrient ecology of *R. fuscipes*, *R. marginatus* and *R. kumarii* fed on different pest which showed a high intake of protein and lipid followed by carbohydrate and in our studies after the feeding on different pest, increased protein quantity of VS was observed in the predator fed with *S. litura* than *H. armigera* and *C. cephalonica*.

2.6. CONCLUSION

We concluded that three-day starved predators in the continuous milking method yield a high quantity of VS. And in this method the animals survive up to 6 days of the starvation period and on the third day the animal secretes the maximum quantity of VS. The prey type also determines the quantity of VS yield and the feeding of *R. fuscipes* with *S. litura* makes them to provide a high quality of VS. Such long fasting periods are a natural situation for reduviid. Different prey types receive different VS quantities according to the difficulty in overwhelming them.