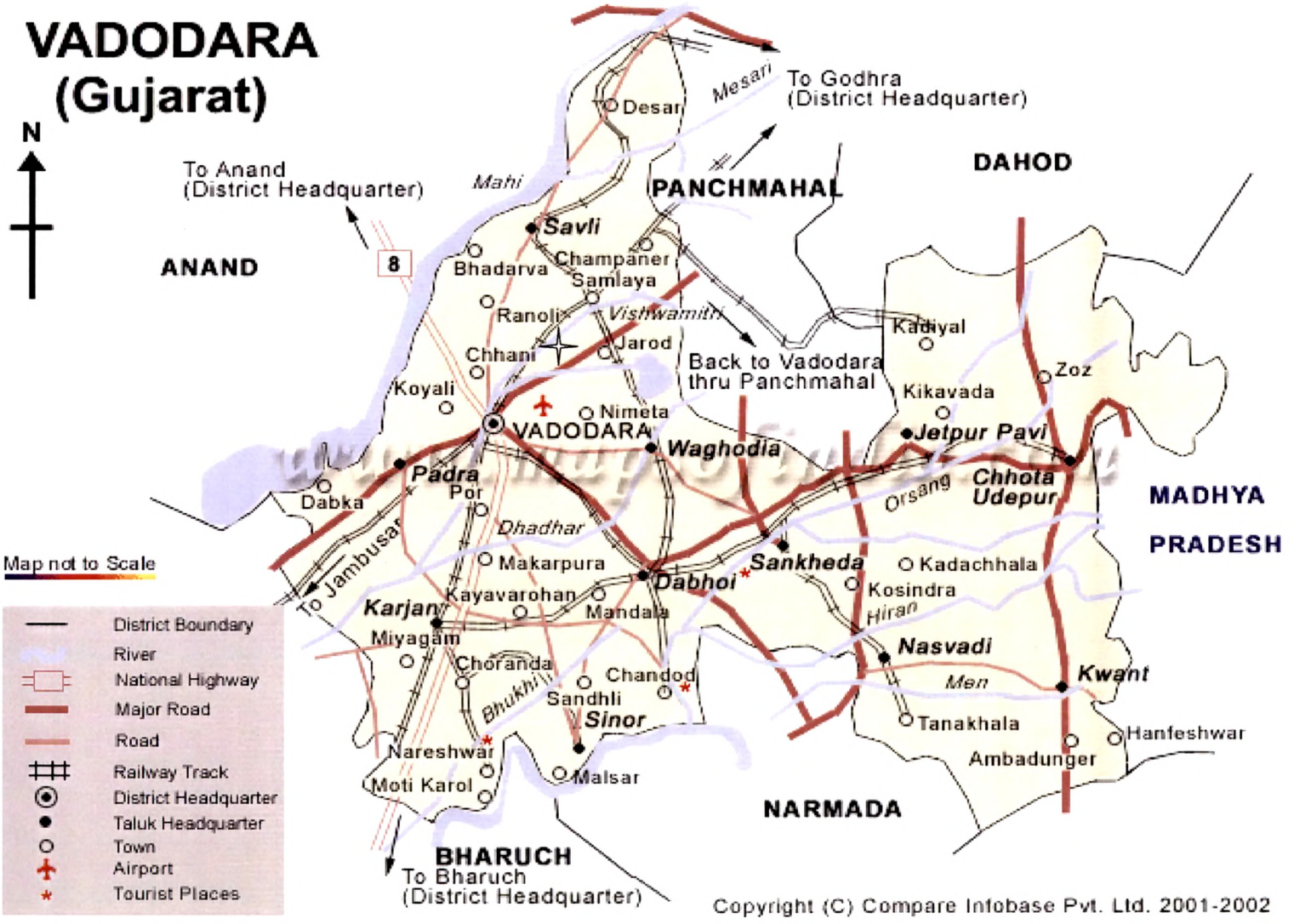
Chapter III – MATERIALS AND METHODS



Location of Study Site

Materials and Methods

During the first year of study field survey was undertaken to know the composition of spider fauna in the study area. Spider fauna of major agroecosystems found in the surrounding areas of Vadodara, central Gujarat in several crops has been undertaken by past workers from our lab (Siliwal, 2002 and Shivakumar, 2006).

A. FIELD STUDIES

Study site: The site of study is located approx. 15 kilometres towards the north east of Vadodara city, in village Hanumanpura (Taluka: Waghodia). The area shows mixed cropping pattern with major crops being Paddy, Cotton, Pigeon pea and Castor. The vegetation in the field margin consists chiefly of shrubs of *Prosopis*, *Acacia*, *Calotropis* and few Cacti. Webs of social spiders are seen commonly in the area along the field margins.

The crops present were scouted on a fortnightly basis for different insect species present in the agroecosystem, to understand the major insect pests present in the cultivated area. The crops cultivated in the study site were Paddy, Cotton, Pigeon pea and Castor. The insect pests were brought to the laboratory and identified under stereozoom microscope (Leica MZ16A) using taxonomic keys and standard monographs Lefroy (1909), Winterblythe (1986) and Borror (1989).

Spiders present in the cultivated area as well as field margins of the agroecosystem were monitored at fortnightly interval in the morning hours between

7:00hrs to 10:00hrs by collecting them manually. The monitoring was done to understand the spider fauna present in the different cropping seasons namely Kharif (June to October) and Rabi (November to April) within the crops area as well as field margins. The spiders were brought to the laboratory and identified under stereozoom microscope (Leica MZ16A) using taxonomic keys and standard monographs. Tikader 1982a, 1982b, Tikader 1987 and Tikader and Malhotra 1980. Photo documentation of the spiders collected was done using Sony still camera with Macrolens (Model: DSC H-1).

A1. Presence of Social Spiders in the Field Margins; Its Diet Composition and Prey Spectrum Studies

The Prey Spectrum studies included the web analysis of the prey remnants. Spiders are fluid feeders performing extra-oral digestion; during their bouts of feeding they leave the exoskeleton of their prey. This exoskeleton can become the vital clues to determine the diet breadth of the spider in agricultural fields.

The webs of the social spider *Stegodyphus sarasinorum* Karsch occurring along the field margins were analyzed at fortnightly interval during morning hours (07:00hrs to 10:00hrs). Webs at three different sites of three different fields located in a radius of 0.5kms were observed. The webs were searched for insect remnants. The remnants were collected and brought to the laboratory for further identification of the prey taxa. In addition to the above while collecting the prey remains, photographs were also taken using Sony DSC H-I Still Camera with Macrolens as photographic evidence. Insect and

insect pests were then identified using taxonomic keys and standard monographs Tikader B.K (1982, 1980, and 1977) and Siliwal (2002). There were few remnants which could not be identified due to desiccation and/or due to damage were not taken into consideration.

Of the several spiders found in an agroecosystem, social spider *Stegodyphus sarasinorum* is found quite commonly and in abundance along the field margins of agricultural crops in Vadodara. Sheets of silk webs were found covering the branches of the peripheral bushes and shrubs of the fields.

The abundance of this species of social spider indicated the prey availability as well as the efficacy of these spiders to survive in the agroecosystem. Thus, they were selected for the present study. Prey spectrum of the spider species is an indicator of its potential as a biological control agent for different pests in the agroecosystem. The study included the field analysis of the prey spectrum of the spiders, to explore its potential as a biocontrol agent. The studies on tolerance of the social spider to pesticide spray drift were carried out in controlled conditions in the laboratory. Hence, Field as well as Laboratory studies were taken up during this study.

B. LABORATORY STUDIES

B1. Effect of Agrochemicals on Spiders: Direct Application

Collection and Maintenance of Social Spider: Colonies of *Stegodyphus sarasinorum* Karsch were collected from field margins manually by cutting the twig of the plant (branches of *Prosopis* and *Acacia*) and were kept in jars of height 30 centimeters (cms.) and diameter 16 cms. The mouth of the container was covered with a muslin cloth and kept at 28°±2°C temperature. The photoperiod was L: D 10:14 and the relative humidity were maintained at 60-70%. Every seven days 2-3 grasshoppers or cockroaches (adults as well as mature nymphs) were given as food. Each jar contained approximately 80 to 100 spiders. Water was sprayed on alternate days in the cage to maintain moisture.

Initially artificial diet for spiders was also tried as suggested by Amalin et al. (2001). However, it was not found acceptable by *S. sarasinorum.*

In field conditions due to wind the spiders get exposed to pesticides through spray drift or residual spray on the web. As a result of the insecticide spray the spiders come in contact with the chemicals via two routes of exposure. (i) Direct Contact (ii) Residual Contact via their Web. In order to simulate the field exposure of the chemicals on spiders, they were subjected to two routes of exposure.

(A) Topical Application: Spiders of uniform size were separated in individual plastic containers of diameter 3.9 cm and height 6 cm. The pesticide/test solution was

applied dorsally on the abdomen of the spider. 2µl (micro litre) of the pesticide /test solution was applied using a micropipette (Tarson's Accupipete, T10).The spiders were allowed to acclimatize for 15 minutes before the application .Observation was taken daily till 72 hours; then the spiders were observed for mortality after 1 week. Cumulative mortality was noted at the end of 1 week.

(B) Vial Coating : Clean vials were coated with 0.5 ml of pesticide solution by manually rolling the vials for 40 to 50 seconds. Then the vials were allowed to dry completely for 8 hours and spider of uniform size were allowed in each vial. Observation was made for mortality after 1 week. Cumulative mortality was noted at the end of 1 week.

For both the exposure routes namely vial coating and topical application, the test was conducted as Complete Block Design (CBD) with 2 replications each for all the five chemicals. Both the replications had 6 test individuals for each treatment. All the five chemicals were first tested at recommended rates and in subsequent tests the dosage/concentration of each chemical was increased or decreased geometrically to obtain LC_{50} and LD_{50}.

The third possible route of exposure is through insecticide exposed prey. Since the effect of the chemical pesticides is fast acting they kill the insect before they can move out of the field. Since these spiders are located along the field margins, they come in contact with the exposed prey at less frequency and hence such a study was not undertaken.

Web Building Potential of the spiders: The web building potential of individual spiders in response to administration of chemical pesticides through two routes were analyzed. Spiders were also treated with plain water via both the testing methods and kept in individual vials. These spiders served as reference for comparison of the webs built by spiders treated with pesticide solution and are referred to as Untreated Control. One week after the exposure of the spiders to the pesticides, the webs built were observed closely and a rating/ranking was given for extent of webs built by the spiders. The spiders showing least effect on web building (which was equivalent to that built by Untreated Control spiders) was given Rank 1 and those spiders showing maximum effect were given Rank 4.

Pesticides: The pesticides for toxicity studies were chosen keeping in mind the broad spectrum of chemicals that social spiders might have exposure to and the popular usage of the chemicals by the farmers.

The spiders were exposed to five pesticides namely;

1. Lannate [Methomyl 40 SP] mfg. by E.I.DuPont Ind. Pvt. Ltd.; this insecticide shows contact as well as systemic toxicity and targets chiefly the Lepidopteran pests like *Heliothis armigera* and *Spodoptera litura.*
2. Tatamida [Imidacloprid 17.8 SL] mfg. by Tata Rallis Agrochemicals Pvt. Ltd.; this insecticide shows contact as well as systemic toxicity and targets chiefly soft bodied insects/ sucking pests. It is used against several insect pests like Aphids, Thrips, Jassids, Whiteflies etc.

3.Ricel [Endosulfan 15 EC] mfg. by Krishi Rasayan Exports Pvt. Ltd.; this is a broad spectrum insecticide used for sucking as well as Orthopteran pests like hoppers. It is a very popular insecticide among Indian farmers and is used in several crops. It is used against sucking pests like whiteflies, aphids, leafhoppers, bugs of Lygaeidae Family and chewing pests like cabbage worms.

4.Econeem [Azadirachtin 10,000 ppm] mfg. by Vallabh Pesticides Ltd.; this is a broad spectrum botanical insecticide promoted by IPM programs and is known to be safe to non-target arthropods. It a prothoracicotropic hormone (PTTH) inhibitor and impairs the growth and development of insects. It is widely used against several sucking as well as chewing pests like Aphids, Whiteflies, Leaf hoppers, Bollworms, Army worm, Weevils etc.

5.Glycel [Glyphosate 5 EC] mfg. by Vallabh Pesticides Ltd.; this is a popular herbicide used for grasses as well as broad leaf weeds. It inhibits synthesis of amino acids tyrosine, tryphtophan and phenylalanine in plants thus inhibiting the growth. Herbicides are slowly gaining popularity with the rising cost of labour and have direct influence on spiders as they directly affect the habitat of the spiders.

All the above chemicals were tested at recommended dosage; the doses were increased or decreased geometrically in relation to the dose dependent mortality obtained for each chemical. (For example:- Recommended dose for Endosulfan 35 EC is 50ml in 10 lit water; the range of dosage tested was 25 ml, 50 ml 75 ml, 100ml and 125ml in 10 lit water. This dose was converted to (parts per million of formulation) ppm formulation for all the subsequent tests.

B2. Effect of Agrochemicals on Spiders: Study on Enzymes as Biomarkers of Toxicity Due to Agrochemicals on Spiders

The Results of Topical Application and Vial Coating experiments showed that these spiders were relatively resistant to most classes of chemical pesticides. Further studies were undertaken to understand the Behavioural and Molecular mechanism involved in the resistance to these chemicals. As it is known that most of the pesticides have a neurotoxic action so Acetylcholine esterase (AChE) levels were compared between control and experimental groups. In insects the detoxification occurs mainly by the enzymes belonging to Glutathione family and hence in the present study, Reduced Glutathione (GSH) and Glutathione S Transferase (GST) were analyzed. GSH contributes to reduction of toxic Lipid peroxides (LPO) and transforms them into non-toxic primary alcohol through oxidoreductase reactions in presence of Glutathione Peroxidase (GPx) enzyme. Formation of lipid peroxides indicates that the integrity of biological membranes is being assaulted or has been compromised. Hence LPO was also studied.

Protein estimation was done using **Lowry *et al* method. (1951)**

AChE estimation was done using **Ellman's method. (1961)**

GSH estimation was done using method described by **Beutlar *et al.* (1963)**

GST estimation was done using method described by **Habig *et al.* (1974)**

LPO estimation was done using method described by **Buege and Aust method. (1978)**

METHOD FOR PROTEIN ASSAY (Lowry *et al* method. 1951)

Reagents Required

1. BSA stock solution (1mg/ml),

2. Analytical reagents:

(a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)

(b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution.

Prepare analytical reagents by mixing 2 ml of (b) with 100 ml of (a)

3. Folin - Ciocalteau reagent solution (1N) Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water)

Principle

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most protein estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml (micro-gram per millilitre) and is probably the most widely used

protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

Procedure

1. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ ml.
2. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well.
3. This solution is incubated at room temperature for 10 minutes.
4. Then add 0.2 ml of reagent Folin-Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660 nm.
5. Plot the absorbance against protein concentration to get a standard calibration curve.
6. Check the absorbance of unknown sample and determine the concentration of the unknown sample using the standard curve obtained by BSA.

BSA (ml)	Water (ml)	Sample conc.(mg/ml)	Sample vol.(ml)	Alk CuSO4 (ml)	Lowery reagent (ml)
0.25	4.75	0.05	0.2	2	0.2
0.5	4.5	0.1	0.2	2	0.2
1	4	0.2	0.2	2	0.2
2	3	0.4	0.2	2	0.2
3	2	0.6	0.2	2	0.2
4	1	0.8	0.2	2	0.2
5	0	1	0.2	2	0.2

METHOD FOR ACETYLCHOLINESTRASE ASSAY (Ellman *et al* method. 1961)

Reagents Required

1) Phosphate buffer (PB), pH 8.0, 0.01M

2) Dithiobisnitrobenzoate (DTNB)

3) Acetylthiocholine Iodide, 0.1M, 0.075M, 0.05M, 0.025M, 0.01M and 0.005M

Principle

This method employs **acetylthiocholine iodide** (ATChI) as a synthetic substrate for AChE. ATChI is broken down to thiocholine and acetate by AChE and thiocholine is reacted with dithiobisnitrobenzoate (DTNB) to produce a yellow colour. The quantity of yellow colour which develops over time is a measure of the activity of AChE and can be measured using a spectrophotometer at 412nm.

These coupled reactions are represented by the following equations:

acetylthiocholine iodide ==========> thiocholine + acetate

thiocholine + dithiobisnitrobenzoate ======> yellow colored products*

* Products of the reaction are 2-nitrobenzoate-5 mercaptothiocholine and 5-thio-2-nitrobenzoate (the latter is the yellow product)

The activity of an enzyme is generally expressed as a rate: the quantity of substrate (in moles) which is broken down by a known amount of enzyme per unit time. In this case, it will be the amount of ATChI which is broken down by AChE per minute.

Procedure

1. Prepare homogenate in 0.05M Phosphate buffer.
2. Add 0.5% Triton – X 100 and 2mM EDTA to the homogenate.
3. Centrifuge at 10,000g at 4°C for 20min. and use supernatant as enzyme source.
4. In a 4ml cuvette, take 2.86ml sod. Phosphate Buffer.
5. Take 0.1ml of supernatant to be used as enzyme and incubate at room temperature for 5 minutes.
6. Add 0.01 ml DTNB solution.
7. Add 0.03 ml of ATChI
8. Record increase in absorbance at 412nm for 30 minutes against blank. OR Incubate for 30min and add 0.1M Eserine and record absorbance at 412nm.

In the present studies, increase in absorbance at 412nm was recorded for 30 min against blank.

METHOD FOR REDUCED GLUTATHIONE (GSH) ASSAY (Beutlar *et al* method. 1963)

Reagents Required

1) Phosphate buffer (PB), pH 7.4, 0.1M

2) Sodiun Citrate (1%)

2) Dithiobisnitrobenzoate (DTNB)

3) Precipitating Reagent

Glacial metaphosphoric acid	1.67 %
EDTA	0.2 %
NaCl	30 %

All the above compounds are dissolved in Re-Distilled Water.

Principle

Glutathione is a major non protein thiol present in the tissue. The sufahydryl group in glutathione reduces the 5, 5 – dithiobis-2-nitro benzoic acid (DTNB) to form one mole of 5-thio-2-nitrobenzoate (TNB) per mole of SH by the following reaction:

2GSH + DTNB ==========> GSSG + TNB

TNB anion has an intense yellow colour with absorbance maxima at 412 nm and can be used to measure – SH group.

Procedure

1. Prepare homogenate; add 1.5ml Precipitating Reagent to 1ml of the homogenate.
2. Keep it at room temperature for 5 min; Centrifuge at 3000 RPM for 15 min.
3. In a 4 ml cuvette to 0.5 ml supernatant add 2 ml phosphate solution and DTNB and record absorbance at 412nm.

METHOD FOR GLUTATHIONE-S-TRANSFERASE (GST) ESTIMATION (Habig *et al* method 1974)

Reagents Required

1) Phosphate buffer (PBS), pH 6.5, 0.1M

2) Reduced Glutathione 50mM in PBS

3) 2, 4 dinitro-chlorobenzene (CDNB)

Principle

Glutathione transferases catalyze the conjugation of 2,4 dinitro-chlorobenzene (CDNB) or 3,4 dichloronitrobenzene (DCNB) with reduced Glutathione (GSH) to produce a yellow product that has an absorbance maxima at 340 – 360nm and the rate of product formation, that indicates the enzyme activity, can be calculated by following the increase in absorbance at 340nm.

Procedure

1. Prepare homogenate in PBS containing EDTA.
2. Keep it at room temperature for 5 min; Centrifuge at 10,000 RPM for 10 min.
3. In a 4 ml cuvette, to 0.03 ml supernatant add 2.77 ml phosphate solution, 0.15ml reduced Glutathione and 0.05ml CDNB.
4. Record absorbance at 340nm.

METHOD FOR LIPID PEROXIDASE (LPO) ESTIMATION (Buege and Aust method 1978)

Reagents Required

1) Phosphate buffer (PBS), pH 7.4, 0.1M

2) Thiobarbuturic Acid (TBA) Reagent (to be freshly prepared)

3) Drabkin's Reagent

Principle

Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with Thiobarbuturic acid (TBA) and gives Thiobarbuturic reactive substances (TBARS). TBARS gives a characteristic pink colour that can be measured colorimetrically at 532nm.

Procedure

1. Preparation of Thiobarbuturic Acid Reagent: TBA - 100mg
   EDTA - 46mg
   20% TCA- 10ml
   2.5 N HCl - 5ml make up to 20ml
2. Prepare 10% homogenate in PBS.
3. In a test tube add the following:

Reagents	Blank	Sample
Homogenate	-	1ml
RDW	1ml	1ml
TBA Reagent	1ml	1ml

4. Keep the test tube in water bath (approx. 95 C) for 20 minutes
5. Cool the test tubes till room temperature
6. Centrifuge at 3000 rpm for 15 min and record absorbance at 532 nm.

For all the above enzyme assays, the spiders were exposed to sublethal dose (LC_{50} and LD_{50}) of the pesticides. After 24hrs, the surviving spiders were weighed using an analytical weighing balance (Sartorius BP - 61) to obtain the appropriate weight of the tissue and a whole body homogenate was prepared for the analytical studies.

STATISTICAL ANALYSIS

LD_{50} in case of contact toxicity & LC_{50} in case of residual toxicity was calculated using Probit analysis (SPSS 7.5). Mortality was corrected and calculated using Abbott's formula. One Way ANOVA were used along with Bonferronii adjustments to find out the differences among the experimental groups using Sigmastat (Systat Software Inc. 3.5/2007). The graphs were prepared using Prism 5.0 (Graphpad Inc.)

Paddy crop at the study site

Shrubs of *Prosopis* at the study site

Collection of spider webs for laboratory rearing and specimen collection of insects trapped in the web

Laboratory rearing of *S. sarasinorum*; mature female with sub-adults in the rearing jar

Topical Application of chemical; droplet seen on the dorsal anterior abdomen of the spider *S. sarasinorum*

Vial Coating Method; *S. sarasinorum* spider released in treated vial