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SPECIFIC BIOMARKER FOR SEXING PUPAE IN GROUNDNUT PEST, *AMSACTA ALBISTRIGA* (LEPIDOPTERA: ARCTIIDEA)

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ABSTRACT

Determination and expression of sex in insects are important not only in basic biology but also in practical applications. The external morphological characters were evaluated for the determination of sex in *Amsacta albistriga* Walk. (Lepidoptera:Arctiidea) during the pupal stage for laboratory studies. Among the various parameters examined, size, length, weight, location of genital pore, anal pore and the ratio of distance between genital and anal pores are found to be reliable criteria for sexing pupae**.** In addition, Storage protein1 (SP1) appropriately called as "female specific protein" could contribute towards the confirmation of the individual's sex. In *A. albistriga*, the SP1 was separated on 10% SDS-PAGE and their identity was confirmed using immunoblot analysis. This paper deals with two issues simultaneously: A simple technique to separate male and female pupae of *A. albistriga* in livable conditions for further behavioral, physiological and molecular studies. The efficient contribution of SP1 as a biochemical marker and its exploitation in reproduction and integrated pest management.

Keywords: anal pore, female specific protein, genital pore, storage protein 1, reproduction

INTRODUCTION

Groundnut (*Arachis hypogea*) is grown over 8.5 million hectares in India, about 85% as a rain fed and 15% as irrigated (Sudheer and Manjula, 2004). In the world's groundnut production, India ranks first even though the yield/hectare is very low. The *Amsacta* species: *A. moorei* Bult. and *A. albistriga* Walk. are notorious pests of groundnut in the Indian states of Karnataka, Punjab, Maharashtra and Tamil Nadu. Full grown larvae of these red hairy caterpillars devour the entire foliage, flowers and growing points. Although the incidence of this pest is sporadic, it does become serious and cause heavy loss of crops (Gibbons, 1980, Singh, 1980). These pests can be controlled by aerial application of insecticides, but environmental concerns have led to use of alternative control measures such as traps, insecticide-treated targets, and sterile insect techniques (Hendrichs et al., 2000). The sterile insect technique involves the mass rearing, separation of male and female, and release of large numbers of sterilized insects, and the regular introduction of sterilized males to effectively suppress the population and under the certain circumstances lead to its eradication (Dowell et al., 2005). The paradigm of this method of pest control has been the eradication of the screwworm, *Cochlimyia hominivorax*, from almost all of Central America (Krafsur, 1998). However, without an adequate biological knowledge, the control program cannot be successfully accomplished (Pedigo, 1996).

In lepidopterans, the males and females are generally separated on the basis of some external morphological characteristics of pupae and adults (Jackson, 1980, Singh, 1995). Generally, males are much smaller than the females and this criterion gives a fair degree of success in sexing laboratory-reared insects, but the distributions of the size indices overlap; so this is a less useful character. Further, the field collected pupae sometimes are different towards the attributes for size and weight as it depends on factors like type of food, climatic condition and parasite infection in nature (D'Amico et al., 2001; Davidowitz et al., 2003). The sexes of *A. albistriga* in adult stages are determined on the basis of color,

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length, width and weight of the moths. In both the sexes, the adult moth is moderate in size having white forewings with brownish streaks and an orange line running along the coastal margin, and the hind wings white with black spots. This black spot is more prominent in appearance in females than males. In the case of male moth, the abdomen is narrow and the genital pore, partially covered with scales. In contrast, in females, the abdomen is broad and the genital pore, quite clearly visible a weighing almost twice as much as the male moth.

The production of insect hexamerins has been demonstrated to show tissue, developmental stage, and in some cases, sex specific profiles in a number of species (Mine et al., 1983; Seo et al., 1998; Zakharkin et al., 2001; Zhu et al., 2002; Jinwal et al., 2002; Ashfaq et al., 2005; Vanishree et al., 2005; Chandrasekar et al., 2008). They are synthesized in the fat body, secreted into the larval hemolymph and taken up by the fat body shortly before pupation. High methionine hexamerins (SP1) are more abundant in females which are typical of lepidopterans (Telfer and Kunkel, 1991; Pan and Telfer, 1996; Chandrasekar, 2006; Chandrasekar et al., 2008, 2009; Chandraskear, 2009, 2012) playing an important role in egg development (Bean and Silhack, 1989; Ryan et al., 1985; Tojo et al., 1980). Moreover, it has long been known that the accumulation and utilization of storage proteins are prominent events linked to the metamorphosis of holometabolous insects. Pupal stores of the methionine proteins average 1.67 times larger than in males (Telfer and Pan, 2003). As it has been genetically determined and developmentally regulated, sex dependency in the expression of SP1 has been noticed in many species of insects (Tojo et al., 1980; Webb and Riddiford, 1988; Ray et al., 1987; Wheeler et al., 2000; Cunha et al., 2005; Chandrasekar et al., 2008, 2009; Chandrasekar, 2009, 2012).

Sex differentiation of the pupal stage of *A. albistriga* has never been described specifically, but a generalized description of pupae of *Amsacta sp*. has been done by Kasyapa et al., (2002). The sex of lepidopterans can be easily determined through dissection, but more efficient techniques for sexing live insects are necessary. Therefore, an attempt was made as necessary to develop an accurate, quick and non destructive method for sexing the insects at the pupal stage of *A. albistriga*, thereby making a contribution to easy discrimination of sex and a better understanding of the pest, thus leading to further molecular studies. In regard to its support, the sexual dimorphism of SP1 expression could act as the ideal sex-specific marker to support the traditional sex determination of *A. albistriga* Walk. Further, it could also be exploited in insect molecular biology for efficient pest management.

MATERIALS AND METHODS

Test insects

Eggs of *A. albistriga* were collected from the field (Ariyalur Dist., Sendurai Village, Tamil Nadu and kept in plastic boxes in the laboratory (8cm height x 10cm length). After hatching, the neonate larvae were transferred to transparent plastic rearing boxes (24cm height x 23cm length) in an environmental chamber and were maintained on leaves of *Arachis hypogea* at laboratory temperature (26.19 ^{0}C ± 0.38 ^{0}C), humidity (>60 %) and a photo period of 12L : 12D. The young larvae were provided with tender leaves and grown up ones with matured leaves of castor (*Ricinus cummunis*). The last instar larvae were reared in larger insect cage (47 cm height x 32cm breath x 47 cm length, two sides with glass and two sides with mosquito mesh). Molting was observed at 12 hours interval, the width of the head capsule and the length of the caterpillars were measured at 24h interval. At the end of the larval stage, the larvae were transferred to a pupal cage (27cm height x 30cm diameter) 3/4th of which was filled with red soil, to allow the larvae to undergo pupation. To analyze sex related differences in pupal characteristics, parameters like pupal length and weight; abdominal length; abdominal width; length of anal and genital pore as well as the distance between anal and genital pore were recorded. This method involves cutting open the cocoons to remove pupae. The location of anal and genital pores and the distance between the pores were observed with the help of a hand lens and Nikon SMZ 1500 (7.5-112.5X) stereomicroscope.

Sample collection and extraction of proteins

Haemolymph was collected from pre-pupal and pupal stages (S1, P0 and P1) through a small incision made at the extremities. Haemolymph

that flowed the wound, without external pressure, was collected in an eppendorf tube, pre rinsed with phenylthiourea (PTU) solution to prevent tyrosinase action. Samples were centrifuged at 3000 rpm for 5 min at 4˚C to sediment heomocyte and cellular debris. Samples were used immediately or stored at 20° C until further use.

Electrophoresis

Protein samples were mixed with the same volume of denaturing buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8). After heating the sample for 3min at 60 ˚C, 25 µl of each sample was loaded on 10% SDS-PAGE. Electrophoresis was performed under denaturing condition (Laemmli, 1970) until tracking dyes migrate to the end of the gel. Gels were stained with staining solution (0.125% Coomassie blue R-250 in 50% methanol and 10% acetic acid) for 6 hours. The stained gels were later destained with 7.5% acetic acid and 30% methanol. Molecular weight standards (Bio-Rad) were run in the parallel lanes. A standard curve, constructed from their relative migration and known molecular weight, was used to estimate the molecular weight of SP1.

Immunoblotting

To investigate the expression of SP1 and determine the molecular mass of subunit of SP1, the gel was transferred to nitrocellulose membranes in Trans-Blots Semi-Dry Transfer Cell (Bio-Rad, Hercules CA) apparatus according to manufacturer's specifications and standard protocol (Towbin et al., 1979). Efficient transfer of proteins was confirmed by Ponceau S staining and Kaleidoscope molecular weight markers (Bio-Rad). The membranes were blocked overnight in 4.0% non-fat dry milk, 25mM phosphate, 25mM acetate buffer, 0.02% sodium azide, pH 7.0 at 4˚C. After blocking, it was incubated with *Hyphantira cunea* SP1 rabbit antiserum (a kind gift from Prof. Seo Sook Jae**,** Geyongsang National University, South Korea) 1:5000 v/v, diluted in the blocking buffer for 2 h at room temperature (RT). Then, the membranes were washed three times in 25mM phosphate, 25mM acetate, 0.02% sodium azide, pH 7.0 and developed with anti-rabbit alkaline phosphatase conjugate antibody according to the manufacturer's methods (Bio-Rad). The proteins were detected by the color developed on addition of the chromogenic substrate solution and photographed.

RESULTS AND DISCUSSION

From the coordinated studies in the field and laboratory it is possible to provide a full description of the life history of *A. albistriga* Walker. The moth emerges from the soil during the onset of monsoon seasons between July-August in South India. The population of this moth greatly fluctuates from year to year on account of seasonal variations of weather. Life stages of the *A. albistriga* are the eggs, five larval instars, the pupa and adult (Fig.1). The larval instars were visually identified on the basis of color changes, differences in size and specific morphological features after each moult, which are shown in the Fig.1. While studying the life cycle, an arrest in development at a specific pupal stage was observed and it is continued up to 9-10 months due to changes in the environmental conditions showing the diapause stage of *A. albistriga.* The diapausing pupae of this pest were in a fixed period of latency during summer season and failed to break diapause in response to temperature at that time. However, during the next monsoon season the diapause period of the pupal stage was terminated and entered into normal metamorphosis. Development was considerably more rapid during the rainy seasons, the entire life cycle for non-diapausic stage averaged about 39 - 46 days whereas in the diapause condition averaged 9-10 months. Therefore, if the environmental condition is favorable, the chosen pest has two generation per year (non-diapause) and for diapause stage there must be only one generation per year.

The sexing was generally carried out at the pupal stage based on the external morphology and sex specific characteristics. In both the sexes, the genital and anal pores were mid ventral in position (Fig. 2a). In the male pupa, length and weight were 1.80±0.210cm and 0.59 ± 0.05g respectively (Table 1) and the genital pore was present on the ninth abdominal segment, which provides the crescent shaped depression on either side (Fig. 2b). In male pupae, this crescent shaped depression was observed all around, excepting the distal end of the genital pore, while the space between the depression and genital pore was convex. Kasyapa et al., (2002) has reported a similar observation in the male pupa of *A. albistriga*.

The length and weight of the female pupa were 2.40± 0.30cm and 1.30 ± 0.33g respectively. The genital pore was located on the eighth abdominal segment just beneath the inter segmental suture between VII and VIII segment (i.e. immediately below the seventh abdominal segment). The anal pore was present on the tenth abdominal segment in both the sexes. Beatrix and Reginaldo (2000) have described a technique to separate male and female pupae of the sand fly, on the basis of parameters such as size, color and external genitalia. The most distinct feature noticed via sex determination was that the distance between the anal and genital opening was greater in the female than in the male pupa of *A. albistriga* (Fig.2b & Table 1) and the ratio between genital and anal pores in male and female was 1: 3.66. All these differences in pupal characteristics were then confirmed on live adults (Fig.3)**.** Differences in the position of genital opening have been used earlier in sexing the pupae of *Ennomos subsignarius* (Solomon, 1962), *Cydia pomonella* (Peterson, 1965), *Heliothis armigera* (Navarajan paul, 1979), *Pludia orichalcea* (Kumar and Geol, 1988), *Etinella zinckenella* (Jaglan et al., 1995), *Aedes atropalpus* (Zakhar, 2001), *Synanthedon scitula* (Leskey and Christopher, 2003), and *Bombyx mori* (Pasca et al., 2004), which supports the present finding.

Insect haemolymph proteins attract a great deal of attention as the biochemical model system (Wyatt and Pan, 1978). SP1 could be one of the most conspicuous sexually dimorphic biochemical characters in insects. Earlier studies indicate that SP1 of *A. albistriga* is Met rich, sex specific protein (82kDa) and the presence of arylphorin rich SP2 in both the sexes (Chandrasekar, 2009, 2012). In the present study, the concentration of SP1 was maximum in the haemolymph of female pupal stages (P0 and P1). A considerable slump in its intensity was observed during the successive days (Fig.4A,B). Consistently, greater intensity of SP2 (76 & 74kDa) polypeptide was observed in the male at the larval - pupal transition (Fig.4C). Immunoblot analysis using anti-SP 1 antibodies evidently revealed the cross reactivity only with the polypeptides in 82kDa region in female pupae (Fig.D), whereas a diminutive faint signal was observed in case of males. This proves that *A. albistriga* is among the lepidopterans whose female pupae store larger quantities of the SP1 than the males. It is synthesized and secreted in large amounts by fat body cells during final instar stage and are sequestered during pre pupal stage (Chandrasekar et al., 2008, 2009). Amino acids transferred from hexamerins to egg proteins can be assumed to pass through general pools and to be subjected to metabolic conversions. (O' Brien et al. 2000; O'Brien et al., 2002). Sexual dimorphism in relation to SP1 was observed in other lepidopteran species (Locke and Collins, 1965; Tojo et al., 1978; Ryan et al., 1985; Haunerland et al., 1990; Burmester and Scheller, 1997; Wheeler et al., 2000).

Our method of sexing pupae was very useful for determining the gender of adults to be used in laboratory experiments, as further research on the physiological, behavioural and molecular studies of the chosen pest is desired, with the purpose of devising novel ways to implement pest management strategies. One such efficient strategy would be the exploitation of SP1, thus blocking many of the diverse strategies of reproduction in adults, paving the way for wide-ranging implications for protein management in lepidopterans. An advantage of this sexing method is that the females and males can be easily separated at the pupal stage, thus little labour is required for such a task. In conclusion, the most reliable characteristics for sex determination in the pupae of *A. albistriga* were the size, length, weight, the location of genital pore and the ratio of distance between genital and anal pores. Since SP1 expression is controlled by sex specific mechanism, it makes it an excellent model for investigations on the basics of sexing insects**.** This could be foci for advances in basic research and it is a simple yet effective diagnostic technique allowing easy and accurate sex differentiation of pupae of groundnut pest *A. albistriga.* Such an approach is also beneficial to select only males in sterile insect techniques for lepidopteran pest control programmes. Therefore, the development of sexing pupae in *A. albistriga*, in which females could be distinguished precisely from males based on morphological and sex specific biochemical characters, shall result in enormous benefits in regard to its future based studies.

Table 1: Morphometric variations in male and female pupae of *Amsacta albistriga* Walk

Characteristic features	Male	Female
Pupa		
Pupal Length (cm)	1.80 ± 0.21	2.40 ± 0.30
Pupal Weight (mg)	0.59 ± 0.05	1.30 ± 0.33
Abdominal length (mm)	5.84 ± 0.12	6.98 ± 0.22
(Post wing pads)		
Abdominal width (mm)	5.99 ± 0.21	8.96 ± 0.10
Length of genital pore (mm)	0.35 ± 0.01	0.37 ± 0.02
Length of anal pore (mm)	0.57 ± 0.03	0.68 ±0.03
Distance between anal & genital pore (mm)	0.41 ± 0.03	1.51 ± 0.05

*** The values are mean ± SE of 10 samples.**

Fig. 1: Life Cycle of *Amsacta albistriga* Walk

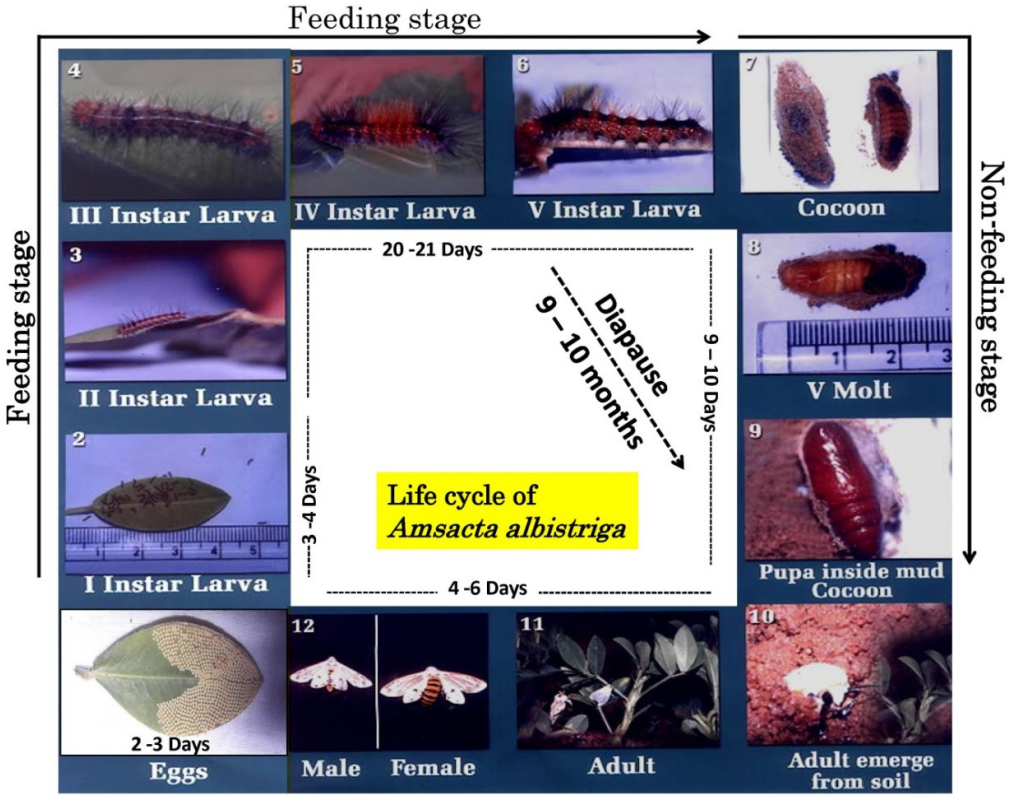


Fig. 2: a) Schematic diagram showing the ventral view of the male and female pupae of *Amsacta albistriga* Walk
b) Ventral view of 7th -10th abdominal segments of the pupae of *Amsacta albistriga* Walk
(ap- anal pore, a- antenna, w- wing, gp- genital pore, ce- compound eyes)

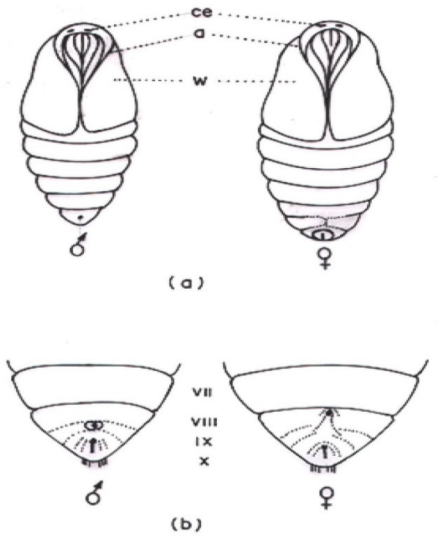


Fig. 3: Male and female adult moth of *A. albistriga* Walk.
a) Pre-pupal stage; b) Pupal stage and c) Adult moth

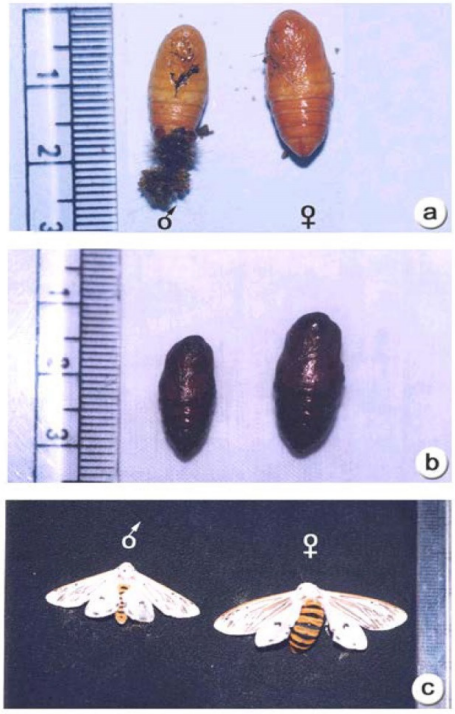
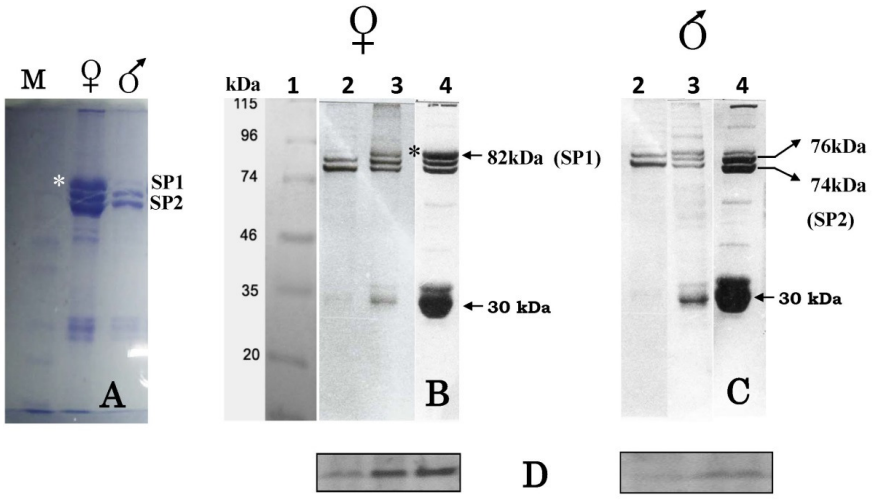


Fig. 4: 10% SDS-PAGE of pupalhemolymph in *Amsactaalbistriga* Walk.
A) Day1 pupal stage;
B & C) Pre-pupa and Pupal Day 0, Day1 stage of male and female hemolymph protein profile
D) Immunoblot
Lane 1: MW marker protein; Lane 2: Spinning day 1; Lane 3: pupa day 0; Lane 4: pupa day 1; SP1-Storage protein 1 (female specific protein); SP2-Storage protein 2;
kDa- kilo Dalton.



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