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Rajiv Gandhi R
Research Scholar, Department of Animal Behaiour and Physiology, School Biological Sciences, Madhurai Kamaraj University, Madurai, Tamil Nadu, India

Meganathan B
Research Scholar, Department of Animal Behaiour and Physiology, School Biological Sciences, Madhurai Kamaraj University, Madurai, Tamil Nadu, India

Sankar R
Assistant Professor, Department of Animal Behaviour and Physiology, School of Biological Sciences, Madhurai Kamaraj University, Madurai, Tamil Nadu, India

Correspondence Author;
Sankar R
Assistant Professor, Department of Animal Behaviour and Physiology, School of Biological Sciences, Madhurai Kamaraj University, Madurai, Tamil Nadu, India

Expression of MHC class II DRB exon 2 gene in *Cynopterus sphinx* during mate selection and estimation of salivary protein

Rajiv Gandhi R, Meganathan B and Sankar R

Abstract
Mate choice or intersexual selection is an evolutionary process in which generally the male mate by a female chooser is dependent on the attractiveness of the phenotypic traits and sexual selection is a mode of natural selection where members of one sex choose mates of other sex to mate with intersexual selection, and compete with members of the same sex for access to members of the opposite sex. Reproductive success is attained by either preferring attractive mates or healthy partners. The phenotypic traits selected by male combat are called secondary sexual characteristics (horns, antlers, etc.) which Darwin described as weapons were selected for this study. We address the effect of female group size on male roosting behaviour in the short-nosed fruit bat *C. sphinx*. We predicted that the time spent by harem males on social grooming by reciprocal licking, tent maintenance, and tent guarding activities would increase with increased female group size. Furthermore, we predicted that the foraging time of harem male bats decreases with increased female group size. Precipitated proteins were purified by FPLC and the preliminary analysis of salivary protein was performed by 2-D gel electrophoresis. Still, standardization of 2-D gel electrophoresis has to be done. MHC class II DRB exon 2 PCR optimization was done using the same primers targeting *Noctilio albiventris* microbat. Even, though *C. sphinx* is the least concerned species, the count of *C. sphinx* is reducing drastically due to several factors. Such as hunting, roost destruction both man and nature, and resource availability. So, we must safeguard this species from poachers and other threats. Every organism is essential for healthy growth and balance of nature.

Keywords: *C. sphinx*, intersexual selection, MHC class II DRB exon 2, salivary protein

Introduction
Mate choice or intersexual selection is an evolutionary process in which generally the male mate by a female chooser is dependent on the attractiveness of the phenotypic traits and sexual selection is a mode of natural selection where members of one sex choose mates of other sex to mate with intersexual selection, and compete with members of the same sex for access to members of the opposite sex. Reproductive success is attained by either preferring attractive mates or healthy partners. Seasonal sexual selection in frogs occurs with the males first gathering at the water's edge and making their mating calls by croaking (Darwin & Wallace 1858) [4]. The females then arrive and choose the males with the deepest croaks and best territories. Currently, five mechanisms explain mate choice: Direct phenotypic benefits, Sensorybias, Fisherianrunaway, Indicator traits, and Genetic compatibility (Dobzhansky *et al*. 1997) [5]. Before copulation, intrasexual selection usually between males may take the form of male-to-male combat. Also mate choice occurs when females choose between male mates (Fisher 1915) [6].

The phenotypic traits selected by male combat are called secondary sexual characteristics (horns, antlers, etc.) which Darwin described as weapons, while traits selected by mate usually female choice are called ornaments. Sexy son theory, good gene hypothesis, fisher runway, and handicapped principle are some theories and hypotheses which explain mate selection.

Cognition, defined as the neuronal processes concerned with the acquisition, processing, retention, and use of information allows animals to track changes in their habitat within their lifetime and solve novel problems, features that seem unequivocally advantageous (Anon n.d.) (Boogert *et al*. 2011) [3].

A role of cognition in attracting mates could in principle be extended to other taxa, if enhanced cognitive skills enable males to acquire more resources, females may obtain direct benefits

from choosing such males as mates; and to the extent that those skills are heritable, they may also benefit indirectly through their offspring. Cognitive specializations such as spatial learning and memory in mammals and particularly song learning in birds have been relatively well studied in a mate-choice context (Light *et al*. 2010; Boogert *et al*. 2011) [7, 3].

Scientists studied how females use male cognitive performance in choosing a mate, 3 types of male behavior that females may assess directly courtship display performance, foraging performance and courtship feeding. Many species rely on chemical signals to attract mates the scent glands, scent-marking behavior and pheromones that transmit chemical signals are often sexually dimorphic (Alcock 2011) [1].

Chemical communication between the sexes occurs in many species: bacteria, protists, fungi, plants and animals all use chemical signals to attract and select mates. There is an enormous diversity of mechanisms mediating chemical communication, and these are often sexually dimorphic for both the sender and receiver. Single-celled organisms and the gametes of multicelled organisms use chemical signals to locate and recognize their mates. To display chemical signals male Danus butterflies uses a pair of elaborate structures called hairpencils, where they court females and position themselves in front of the female. Likewise male blue crabs (*Callinectes sapidus*) perform their elaborate courtship display; they use their swimming legs to waft odors towards attentive females. And the female lampreys (*Petromyzon marinus*) are attracted to testosterone and other odorants in a male's urine. The elaborate courtship behavior of salamanders, such as *Notophthalmus viridescens*, is centered on males rubbing or fanning odors secreted by various 'hedonic' glands onto a female's nostrils or body. Male lizards, such as *Iguana iguana*, release sexual odors through large, femoral glands, whose secretions reveal much information, including their androgen levels and dominance status. In mammals, male display their scent for females using complex mixtures of odorants secreted by a diversity of androgen-dependent scent glands. A male's odor has remarkable effects on a female's reproductive physiology and behavior, such as accelerating puberty, activating ovulation, accelerating and synchronizing estrus, and inducing pregnancy block (Andersson 1994) [2] (Steiger & Stökl 2014) [10]. Male house mice (*Mus musculus*) scent-mark with urine and females are attracted to these marks. Commensal microbes play an important role in shaping an individual's odour. Infection might also trigger immunological responses that alter an individual's odour by interacting with the microbes. For example, the highly polymorphic genes of the major histocompatibility complex (MHC) control immunological self/nonself-recognition and also influence individual odour and mating preferences in mice. Females detect chemical cues from the immune system or stress responses and are capable of detecting the odour of infected males. It may be because the attractive odorant is absent or not able to produce it (Andersson 1994) [2].

A prediction of the pathogen-mediated mechanisms of MHC-dependent mating preferences could increase the production of offspring with enhanced disease resistance. At the level of individual choice, individuals may choose mates based on whether they carry good genes. Alternatively, individuals choose mates with the most compatible genes to their own, to provide offspring with the most adaptive gene combinations (Wobst *et al*. 1999) [11].

Chemo sensation, which is one of the most primitive senses, has evolved into a specialized sensory system. Humans can not only detect, but also assess, and respond to environmental (chemical) olfactory cues especially those used to evoke behavioural and sexual responses from other individuals, also known as pheromones. Pheromones function to communicate one's species, sex, and perhaps most importantly one's genetic identity. The genes of the MHC provide the basis from which a set of unique olfactory coding develops (Penn & Potts 1999; Yamazaki *et al*. 1999) [9, 12].

MHC-based sexual selection is known to involve olfactory mechanisms in vertebrate taxa such as fish, mice, humans, primates, birds, and reptiles (Mayer & Brunner 2007) [8]. At its simplest level, humans have long been acquainted with the sense of olfaction for its use in determining the pleasantness or the unpleasantness of one's resources, food, etc. At a deeper level, it has been predicted that olfaction serves to personally identify individuals based on the genes of the MHC (Fisher 1915; Mayer & Brunner 2007) [8, 6].

Self-grooming is common among terrestrial mammals and serves several functions one of these functions involves anointing the body with salivary, anogenital and other body odours to advertise individual identity reproductive condition and sexual attractiveness. Thus, self-grooming may be a sexually selected trait used in reproductive competition and sexual attraction (Andersson 1994; Alcock 2011; Boogert *et al*. 2011) [1, 2, 3].

Fig 1: *C. sphinx*

Kingdom: Animalia
Phylum: Chordata
Class: Mammalia
Order: Chiroptera
Family: Pteropodidae
Genus: *Cynopterus*
Species: *sphinx*

Despite the huge diversity of about 1300 bat species across the globe (Fenton and Simmons 2014) [13], social grooming has been only reported in about 12 species, *viz*. *Phyllostomus hastatus, Carollia perspicillata, Eidolon helvum, Rousettus aegyptiacus, Myotis bechsteinii, A. jamaicensis, Noclitio lepronius, Pipistrellus kuhlii, Desmondus rotundus, D. ecaudata, D. youngi*, and *C. sphinx*, and the probable functions of this behaviour are suggested to be related to hygiene and social bonding (Wilkinson 1987; Carter and

Wilkinson 2013; Rathinakumar *et al*. 2017) [14, 15]. *C. sphinx* is a medium-sized (40−70 g) pteropodid bat with a harem-type social organization, which is distributed throughout the Indian subcontinent (Bates and Harrison 1997; Storz and Kunz 1999) [17, 19]. Tent-making is exclusively performed by males in these bats. Harem males construct tents on a variety of plants such as *Vernonia scandens*, *Borassus flabellifer*, *Caryota urens*, and *Polyalthia longifolia* and guard their tents and female roost mates against intruding males (Balasingh *et al*. 1995; Storz *et al*. 2001) [18, 20]. However, there is a paucity of information on the time invested by harem males in defending their tent and female roost mates during the breeding season. Therefore, in this study, we address the effect of female group size on male roosting behaviour in the short-nosed fruit bat *C. sphinx*. We predicted that the time spent by harem males on social grooming by reciprocal licking, tent maintenance, and tent guarding activities would increase with increased female group size. Furthermore, we predicted that the foraging time of harem male bats decrease with increased female group size.

Materials and Methods

This study was carried out on the foliage tent roosts constructed by the short-nosed fruit bat *C. sphinx* in mast trees (P. longifolia) during their breeding season (March-November 2021) in the Alagappa University campus (10°4′43″ N; 78°47′40″E), Karaikudi, Tamil Nadu, India. We located about 120 P. longifolia trees on this campus and among them, 20 trees were recorded with tent roosts, at the heights of 4−6 m from the ground, constructed by *C. sphinx*. However, among the 20 tents, only 13 were occupied by these bats while 7 tents remained vacant. We chose five tent roosts with five different female group sizes (*viz*. T10 4, T11 2, T12 7, T14 3, and T15 1), solely based on different female group sizes within the study site (Table 1). Focal observations were made with the aid of a tripod-supported digital camera (Nikon CoolPix P510) and a redfiltered torch light. Tent observations were carried out from dusk to dawn (1730−0630 h) for 30 alternate nights. A single tent was exclusively observed in a night; thereby, all five tents with five different female group sizes were observed sequentially until six observations were obtained from each tent. Observations were carried out on three important aspects of roosting behaviour of *C. sphinx*, namely (i) social grooming (time spent on reciprocal licking before emergence), (ii) tent maintenance (time spent on severing stems and leaves, cleaning the inner surfaces of the tents by licking and pasting of chewed leaves), and (iii) tent guarding (presence of harem male bats perching in its tents either being vigilant by shaking their wings and displaying thumb claws or by maximizing their stay by using tents as feeding roosts or resting). We counted the number of times harem males fly out of their tent per night. The absence of harem male bats from their respective tents was assumed to be foraging time and defined as Btime spent away from tent. ^ The time invested by harem male bats on these social activities was quantified and analyzed with respect to the female group size. A Spearman's correlation test was used to test the influence of female group size on social grooming by reciprocal licking, tent maintenance, and tent guarding behaviors. Similarly, emergence and return time of the bats with reference to female group size was analyzed using Spearman's correlation test (Zar 1999). Spearman correlations based on averaged value per roost (n = 5 roosts) to account for the non-independence of observations per roost gave the same qualitative results throughout. All the values are represented as mean ± SE. All statistical analyses were performed with PASW® statistics (version 18.0).

From the collected salivary samples, protein precipitation was done by adopting the protocol of *Jessie et.al*, 2008. After overnight precipitation using 20% w/v TCA, 90% v/v acetone and 20mM DTT, the crude precipitate were in 10% SDS gel to check for the presence of any protein in the saliva in the saliva of crude and saliva precipitates of 5 male salivary samples.

Purification by FPLC

A Pharmacia (Piscataway, NJ) FPLC chromatography system was utilized for native protein purification. All columns were run at ambient temperature, using Milli-Q filtered water (Millipore, Bedford, MA) and buffer solutions were degassed and filtered through 0.22 pm, cellulose acetate membrane filters (Corning, Corning, NY). Protein solutions, prepared as described above, were further concentrated by ultrafiltration, utilizing membranes having nominal molecular mass cutoffs of 10,000 daltons (PM-10, Amicon Corp., Danvers, MA) to achieve a protein concentration of 1-2 mg/ml. Aliquots of the concentrated supernatants were Superose 12 analytical column injected in 0.5 ml amounts onto a 1 x 30 cm) previously equilibrated with 60 mM sodium phosphate buffer, pH 6.1. Development of the column was completed using the same buffer in an isocratic mode. Fractions of 1 ml were collected and assayed for enzymatic activities. Appropriate fractions were pooled and concentrated by PM-10 ultrafiltration. Samples were also analyzed by SDS Polyacrylamide gel electrophoresis to facilitate quantitation and to complement identification provided by enzymatic assay protocols. Concentrated protein solutions were subsequently injected without further equilibration or treatment in 0.5 ml aliquots onto a 0.5 x 5 cm anion exchange Mono Q column (Pharmacia, Piscataway, NJ), equilibrated in the same buffer as before. The desired proteins were eluted using a linear gradient from 0-1 M NaCl in the same buffer. Fractions were assayed for enzymatic activity as before, and subjected to SDS polyacrylamide gel electrophoresis to verify protein localization.

Isolation of Genomic DNA

DNA of 10 *C. sphinx* individuals (4 – males, 6 – females) was isolated from the wing tissues of 10 animals and they were resolved on 1% agarose gel. Fig 4.30 shows the presence DNA in all the 10 samples. After isolation, samples were quantified and stored. PCR amplification was done with all samples after DNA isolation for semi-preparative PCR assay.

Results and Discussions

Behavior Analysis

Arresting behaviour

We have observed the roosts of *C. sphinx* in the *Borassus flabellifer* tree near Madurai Kamaraj University (Latitude N 9° 56.51', longitude E 78° 00.39008') (Fig 2). Five tents consisting of solitary and harem-forming males. The total social interactions of *C. sphinx* were recorded during mating season. The roost identification was done by observing the shrubs below the palm trees. The ad libitum sampling helped to locate the number of tents and activity patterns.

Fig 2: Map image of the study area

A total of 324 scans were done during field sampling in each tent. The behaviour exhibited by bats was categorized into exhibited behaviours such as AG-auto grooming, and RA-resting alert. A-arresting, GB- grooming ball, TP- tent peeping – male bats flies into other bats tent, CF- circling flight, V- vocalization, HG- hugging, WF- wing fanning, TM-tent marking. Results of this study show that during mating season the male *C. sphinx* exhibited various behavioural repertories, which might have a role in mating. The solitary males usually circle the harem and vocalize by a producing sound like a "chip chip" as an acoustic signal to the mates.

During my observations we have observed circling behavior 22 times, tent peeping 18 times and vocalization 4 times. Grooming ball is commonly observed before foraging this behaviour may have a role in social bonding, the efficiency of flight during foraging, hygienic, and courtship signals (Fig 4.12, 4.13&4.16)

Arresting, hugging and wing fanning are other prominent behaviors exhibited by male *C. sphinx* observed to attract the female *C. sphinx*. The male *C. sphinx* fans its wing after auto grooming, this might have a role in chemical communication. Male bats as a strategy of preventing female bats not to flying away from the tent the male bats arrest the females by covering them and hugging behaviour may have a role in increasing the bonding between the male-female relation in bats this behaviour is often seen after arresting. Scent marking behaviour is used for mate selection in other mammals. So, in *C. sphinx* scent marking might also have a role in mate selection. Resting alertness behaviour is seen throughout the study, the peculiarity of this behaviour is *C. sphinx* move their ears to detect any vocalizations produced by other bats and danger. Auto grooming is seen most of the time, in this behaviour the *C. sphinx* will lick its wings and body. This behaviour might help the bat to clean their body.

Before wing fanning, auto grooming was often observed. This might be part of olfactory communications. During auto grooming, the saliva is licked all over the body of the male *C. sphinx*. So the presence of pheromones, odorant binding proteins, and other volatile compounds in saliva might be dispersed during wing fanning to attract the female. During my field study, due to environmental problems, one tent of *C. sphinx* was damaged Due to constant disturbance by the poachers the activity pattern of *C. sphinx* is highly disturbed. Results were also shown statistically in Figure 3-7.

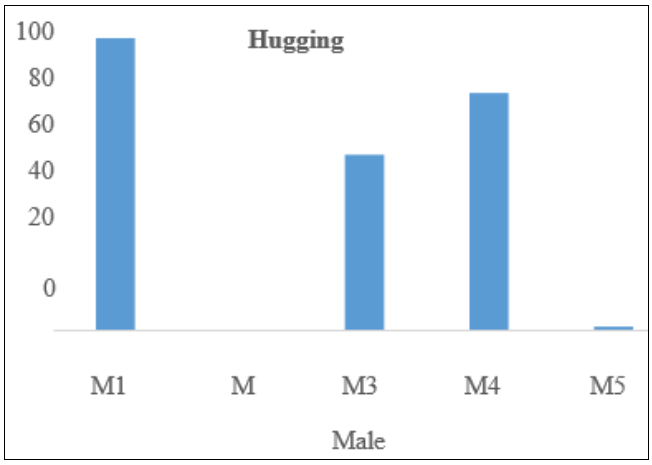

Fig 3: Hugging behaviour of male bats

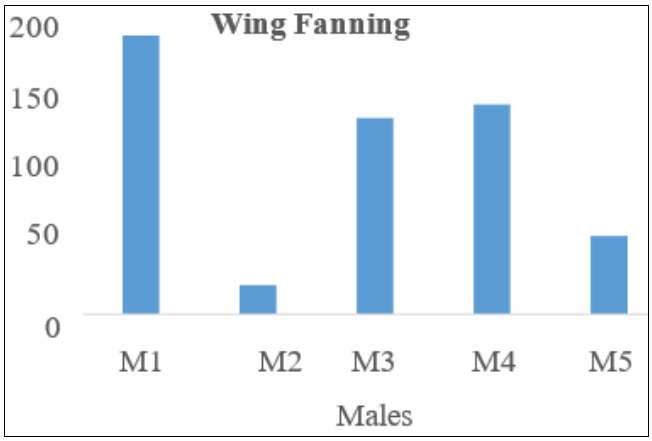

Fig 4: Wing Fanning behaviour of male bats

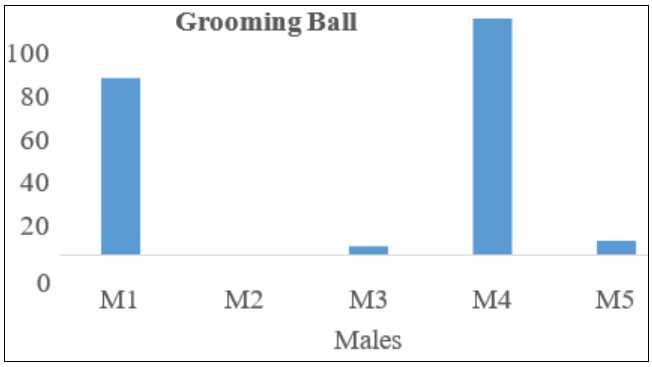

Fig 5: Grooming Ball behaviour of male bats

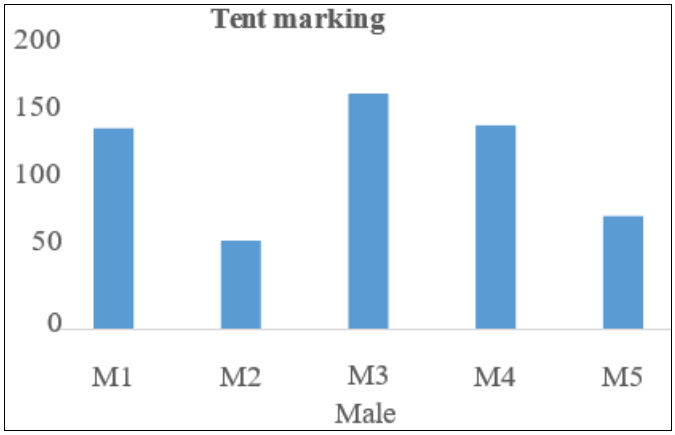

Fig 6: Tent-making behaviour of male bats

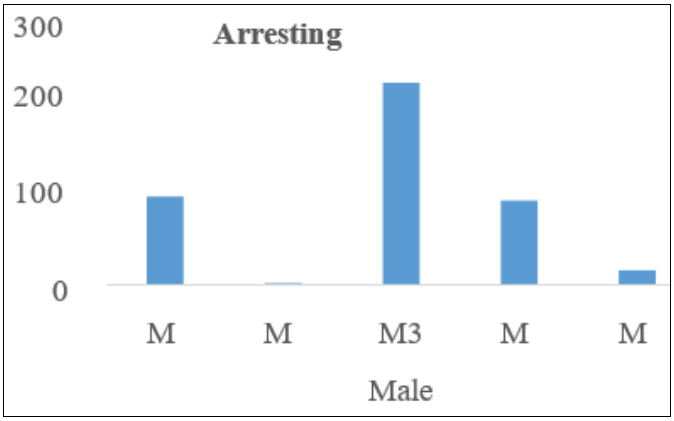

Fig 7: Arresting behaviour of male bats

Proteomic Analysis

1. Standardization of Protein precipitation

From the collected salivary samples, protein precipitation was done by adopting the protocol of Jessie *et al.*, 2008. After overnight precipitation using 20% w/v TCA, 90% v/v acetone and 20 mM DTT, the crude precipitate was in 10% SDS gel to check for the presence of any protein in the saliva in the saliva. The fig 4.18 below shows the resolved gel containing crude precipitates of 5 male salivary samples. After precipitating the sample, the protein was quantified by Nanodrop (Table 1).

Table 1: Results of Nanodrop

Sample	Quantified value	OD 260/280
Blank (Rehydration buffer)	100 mg/ml	
Sample I	103.211 mg/ml	0.11
Sample II	104.578 mg/ml	0.13
Sample III	103.172	0.13
Sample IV	101.846	0.10
Sample V	100.693	0.12

The crude precipitates were then subjected to purification and the purified samples were then resolved in 10% SDS gel. Fig 8A shows the resolved proteins after purification. The banding patterns indicate good purification efficiency of the adopted protocol. With this the protocol for precipitating the salivary proteins from the *C. sphinx* is standardized. Even though the precipitation method is standardized, the commassie blue staining method was not that effective. So, the staining method for the precipitated protein was standardized by silver staining. (Fig 8 B) In silver staining, the even trace quantity of proteins was visualized compared to the commassie blue staining. Silver staining is fast and the accuracy of the results was 50 times greater than commassie blue staining. So, the further steps were also standardized with the help of silver nitrate staining.

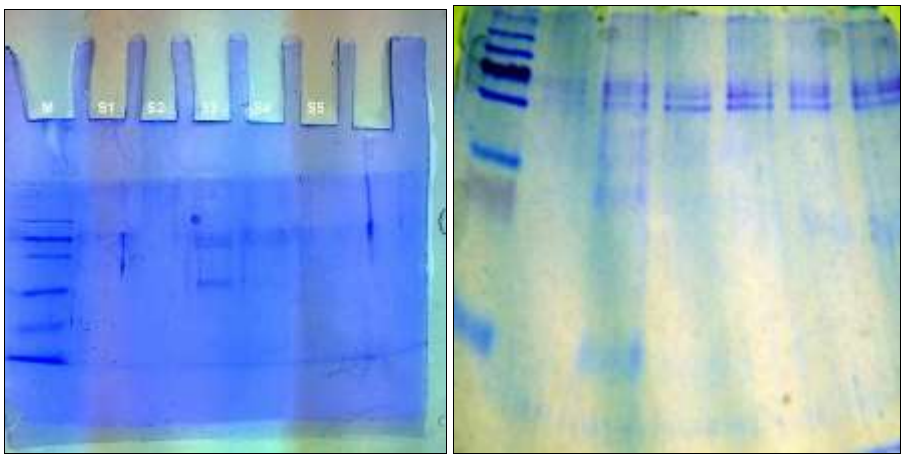

Fig 8A: Gel image of crude saliva 8B. Gel image of digested saliva

Purification of male salivary protein by FPLC

After precipitating the salivary protein, the sample was purified by Fast protein liquid chromatography (FPLC). Using DEAE ion exchange pre-packed column. After running the precipitated sample clear peak appeared in between 0-1M gradient lane. (Fig 9A) The fraction of the peak was collected through a fraction collector. The collected sample was concentrated by a vacuum evaporator. The concentrated sample was run by SDS-PAGE and 2 bands were seen in the range of 46-30 k DA for the male sample. (Fig 9B).

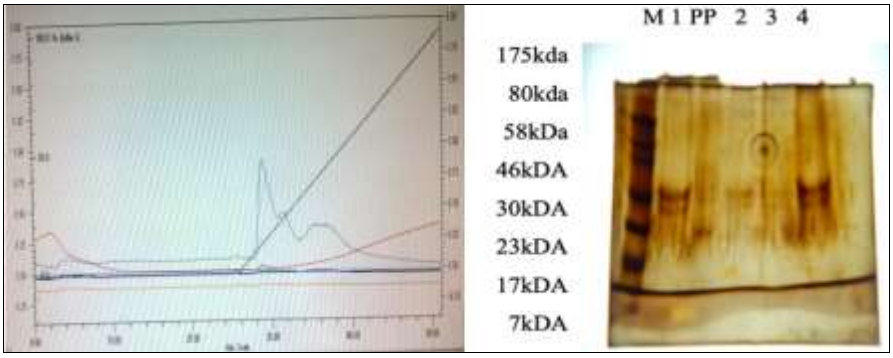

Fig 9A and B: FPLC chart and SDS-Page result of crude saliva sample Lane 1- marker, Lane 2- purified sample 1, Lane 3- protein precipitate, Lane 4- purified sample 2, Lane 5-purified sample 3, Lane 6- purified sample 4

The female saliva was precipitated and purified DEAE cellulose pre-packed ion exchange column. After running the precipitated sample clear peak appeared in between 0-1M gradient lane. (Fig 10A) The fraction of the peak was collected through a fraction collector. The collected sample was concentrated by a vacuum evaporator. The concentrated sample was run by SDS-PAGE and 3 bands were visualized in the range of 46- 23 kDA for the male sample. (Fig 10B) In both male and female samples, the bands present in 46kDA and in female and male one band below 30 kDA is visualized which might be an odorant binding protein, not yet confirmed, should be further confirmed by LC-MS/MS.

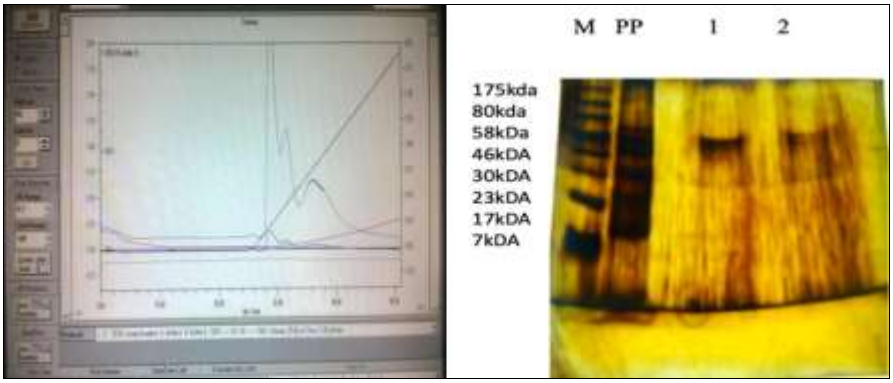

Fig 10A and B: FPLC chart and SDS-Page result of digested saliva sample Lane 1- marker, Lane 2- purified sample 1, Lane 3- protein precipitate, Lane 4- purified sample 2, Lane 5-purified sample 3, Lane 6- purified sample 4.

Proteomic approach to study the chemical communication of *C. sphinx* by 2-dimensional gel electrophoresis

Female salivary proteome: In the female sample, only a few spots were able to be visualized. The proteins are approximately around 60 to 40 kDA and pH 5 to 7. And some spots are seen at 130 kDA and pH 6 to 10. (Fig 11) As the experiment was not standardized, the results were not conclusive.

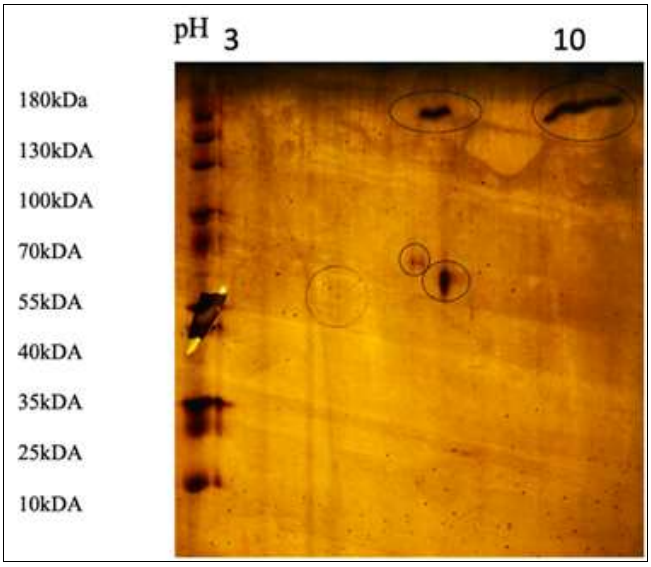

Fig 11: Salivary proteins of female *C. sphinx* in 2D gel electrophoresis.

Male salivary proteome

As the preliminary study of salivary proteome analysis of *C. sphinx*: 2 – 2-dimensional gel electrophoresis was performed, 11 spots were visualized. Manually the molecular weight and PI of the spots were calculated. Molecular weight by PI was compared with already known or sequenced proteins from the saliva of mammal protein in research articles. So as a preliminary work, we theoretically predicted the proteins. It should be further confirmed by standardization of 2D-gel electrophoresis and LC-MS/MS (Fig. 12).

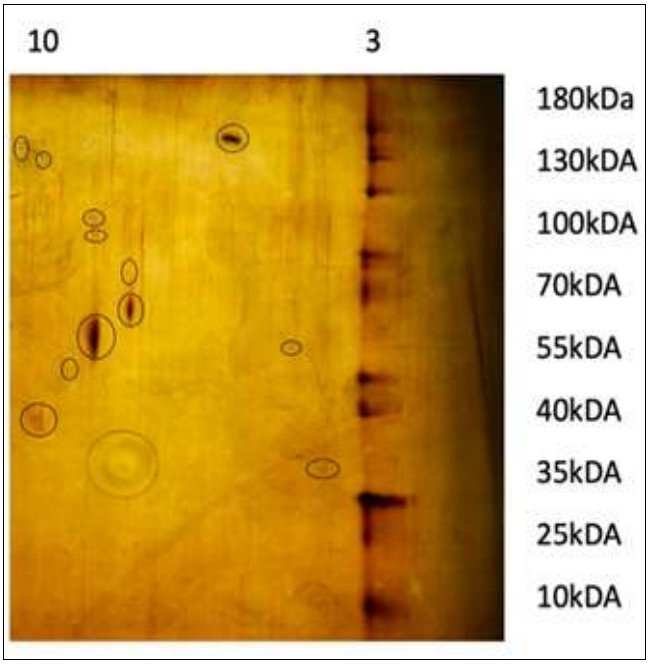

Fig 12: Salivary proteins of male *C. sphinx* in 2-D gel electrophoresis

Theoretical prediction of salivary proteins of male *C. sphinx*

Theoretically, the proteins were predicted (Hu *et al*. 2011) (Table 4.2). Still, we have to standardize further and proteins sequence the salivary proteins of *C. sphinx*. So, we can understand the proteins which are present in saliva are responsible for chemical communications and for other various functions.

Table 2: Theoretical prediction of salivary proteins of male *C. sphinx*

Spot. NO	Theoretical MW/PI	Predicted protein	Accession number	Reviewed MW/PI
1	150/4.3	Un named protein	CAD34875	143.897/4.99
2	**78/8.6**			
3	75/8.4	Hypothetical protein	T17221	72.783/9.47
4	50/7.4	Alpha amylase salivary precurssor	PO4745	57.168/6.47
5	45/8.4	Phospho glycerate kinase	Q9H107	44.665/8.74
6	40/9.2	Hypothetical protein	Q9BRQP	40.501
7	34/9.5	Un named protein	Q98HJG1	62.783/6.47
8	30/3.2	Un named protein	P0HINI3	79.783/8.47
9	60/8.1	Un named protein	VIT09A1	120.492/9.87
10	135/10	Hypothetical protein	Q8N318	130.492/9.87

Molecular Analysis

MHC class II DRB exon 2 gene Amplification

Genomic DNA isolation

Genomic DNA of 10 *C. sphinx* individuals (4 – males, 6 – females) was isolated from the wing tissues of 10 animals and they were resolved on 1% agarose gel. Fig 4.30 shows the presence of DNA in all 10 samples. After isolation, samples were quantified and stored. PCR amplification was done with all samples.

PCR standardization

Repeated trials with different primer pairs, and various PCR conditions were carried out to amplify MHC DRB exon 2. Among various primers, Int 2a and Int 1b were showed good amplification of MHC class II DRB exon. Since the primers are from a microbat (*N. albiventris*), standardization of annealing temperature was done. The amplicons obtained at different gradients (56-60̊ C). Non- specific amplification was observed with amplicons sizes 750 bp, 350 bp and 1.5 kb. After standardization, 350 bp amplicon was observed. The actual size of exon 2 is not yet determined for *C. sphinx.* But for other bat species, exon 2 is reported around 300bp length.

MHC class II DRB exon 2 gene Amplification

After standardization of PCR to check for the MHC-based mate selection in *C. sphinx* Single stranded conformation polymorphism was done. Some Variations in the strand were visualized. (Fig 13A and 13B) Still, it should be standardized further by increasing the sample size and by performing Denaturing gradient gel electrophoresis. It should be confirmed by sequencing from eluting the bands showing variations. As MHC plays a prominent role in mate selection, studying and confirming the variations in MHC DRB exon 2 will give more information about mate selection in genetic level of *C. sphinx.*

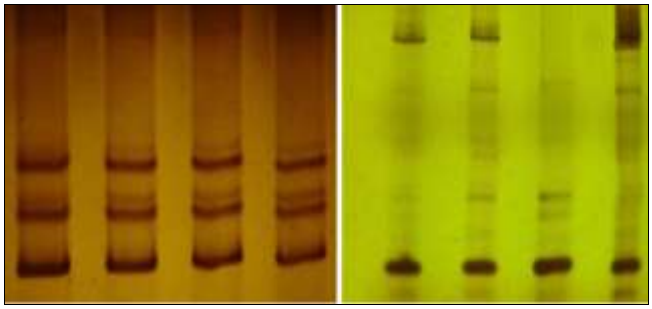

Fig 13A and B: MHC-based mate selection in *C. sphinx*, Single strand conformation

Conclusion

Various social interactions were observed during mating season such as wing fanning, scent marking, social grooming, arresting, and hugging, circling flight and ten peeping which might have a role in chemical communication and mate selection. Male bats vocalized around the tents of other bats by producing unique acoustic signals. The protocol for the Precipitation of Salivary proteins was standardized by using both silver staining and coomassie blue. Precipitated proteins were purified by FPLC and the preliminary analysis of salivary protein was performed by 2-D gel electrophoresis. Still, standardization of 2-D gel electrophoresis has to be done. MHC class II DRB exon 2 PCR optimization was done using the same primers targeting *Noctilio albiventris* microbat.

Preliminary analysis of mate selection in *C. sphinx* was done by single-stranded conformation polymorphism. Still, the experiment protocols had to be optimized. During my field study, we observed the disturbance of *C. sphinx* roost due to local people hunting and the tents were frequently damaged due to sudden environmental changes. Even, though *C. sphinx* is the least concerned species, the count of *C. sphinx* is reducing drastically due to several factors. Such as hunting, roost destruction both man and nature, and resource availability. So, we must safeguard this species from poachers and other threats. Every organism is essential for healthy growth and balance of nature.

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