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Glandular secretions of male *Pteropus* (flying foxes): Preliminary chemical comparisons among species

By Jamie Wagner

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1. Abstract

Chemosignaling – passing information by means of chemical compounds that can be detected by members of the same species – is a very important form of communication for most mammals. Flying fox males have odiferous marking secretions on their neck-ruffs that include a combination of secretion from the neck gland and from the urogenital tract; males use this substance to establish territory, especially during the mating season. The secretions of flying fox males from three Australian species – spectacled (*Pteropus conspicillatus*), grey-headed (*P. poliocephalus*), and black (*P. alecto*) – were compared using high pressure liquid chromatography (HPLC); two spectacled females were also examined to compare secretion content without the addition of urogenital components, as female neck-ruff secretions originate solely from the neck glands. Male secretions showed five to six major components, and each species demonstrated a unique chemical profile. Further, female secretions revealed a greater volume of components than expected, though male secretions contained more major components that were generally at greater concentrations. It was found that spectacled, grey-headed, and black flying fox secretions had many shared components, which may be related to the ability of black flying foxes to interbreed with the other two species. Further examination is needed to determine the component identities, though this study hypothesizes them to be alcohols, esters, hydrocarbons, or ketones based on these compounds' unique odors and common abundance in mammalian secretions.

Key words: flying foxes, marking secretions, high pressure liquid chromatography, neck gland, urogenital

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4. Introduction

4.1. Flying foxes

4.1.1. Megachiroptera

Bats are flying nocturnal mammals in the order Chiroptera, which includes the sub-orders Megachiroptera (megabats) and Microchiroptera (microbats) (Hall and Richards 2000). Pteropodidae – with an Australasian and African distribution – is the sole family of the megabats, while microbats encompass seventeen families and are found on every continent except Antarctica. The similarity in wing structure between these two groups is a case of convergent evolution, as this outer form is most efficient for flying mammals. These bats are greatly separated evolutionarily; the microbats most recently shared a common ancestor with shrews, while megabats are most closely related to Madagascar lemurs and are actually considered primates (Hall and Richards 2000). Consequently, these sub-orders have very different characteristics. Microchiroptera are primarily carnivorous and insectivorous bats, often with poor eyesight, that rely primarily on echolocation to navigate. In contrast, Megachiroptera are frugivorous (fruit-eating), nectivorous (nectar-eating), and pollen-eating bats that navigate by means of their acute vision and highly developed sense of smell. In general, megabats are larger than microbats, although this is not true in all cases.

4.1.2. Australian Species and Distribution

Australia has seven species of megabats in the genus *Pteropus* (Hall and Richards 2000). These bats are known as flying foxes because their large size and pointed muzzles give the bat's head a fox-like shape. The four most common species in Australia are *P. conspicillatus* (spectacled flying fox), *P. poliocephalus* (grey-headed), *P. alecto* (black), and *P. scapulatus* (little red) – all found in the eastern, northern and western forested edges of the Australian continent (Figure 1). The other three flying fox species are limited to individual

islands off the Australian coast. *Pteropus* inhabit coastal tropical to temperate forests, mainly in rainforests but also including mangroves, swamps, and tall open forests, though always within a few kilometers proximity to rainforest (Churchill 1998). Spectacled flying foxes, named for the rings of light-colored fur around their eyes, have the smallest range of the continental Australian species, from Cadwell to Cooktown with a disjunct population in the Cape York Peninsula Iron Range (Fox et al. 2008). The grey-headed flying foxes, found from Maryborough in Southern Queensland to Melbourne, are the only species with fur extending all the way down to the toes (Churchill 1998). The largest of the Australian species is the black flying fox, one of the biggest bats in the world with a wingspan up to 1.5 meters. Blacks also have the largest distribution in Australia, from northern New South Wales, up the eastern part of Queensland, across the top of the Northern Territory and Western Australia, and down to Carnarvan. Little reds are the smallest of these four *Pteropus* species, but have the greatest range. They extend from Melbourne northeast across northern Australia and down to Carnarvan, as well as extending furthest inland of all the continental species.

Figure 1: Distribution Map of Australia's Flying Foxes

Map of the distribution of Australia's four mainland species of flying foxes. Created using Churchill 1998, Fox et al. 2008, and Hall and Richards 2000 as references.

4.1.3. General Life History

Though flying foxes are considered nocturnal, they are also active for short periods throughout the day, alternating between napping and socializing within their roost camps (Hall and Richards 2000). The camps can be extensive, with thousands of bats hanging from the branches of mature rainforest trees. At dusk, they leave the camps to feed, returning in early dawn; the preferred food for flying foxes is blossoms (nectar and pollen), followed by rainforest fruits and occasionally leaves. During feeding times they typically cover between 4 and 30 kilometers, but often up to 50 kilometers, in a single night (Churchill 1998). Flying foxes play a critical role in Australian forest ecosystems as pollinators and as one of the primary seed distributors for a significant number of rainforest fruits (Hall and Richards 2000).

A female flying fox gives birth to a single offspring per year and does not reach sexual maturity until age two (Hall and Richards 2000). Female flying foxes carry their offspring for about three weeks, after which the young are too heavy to carry and are left in crèches within the colony trees during the mothers' nighttime forages. Maternal care continues for about four to five months, during which time the juveniles learn to fly and then begin to explore and forage. The average lifespan of wild flying foxes is six years (Fox et al. 2008).

4.2. Previous Research

4.2.1. Male Reproductive Anatomy and Territoriality

Some of the major features of the male reproductive system include two testes (Appendix A, Figure A1), the penis (Appendix A, Figure A2), and a small prostate gland (Hall and Richards 2000). Flying foxes also possess pairs of highly specialized sebaceous (oily) glands located on the neck-shoulder region (Spencer and Flick 1995, Wood et al. 2005). Other gland sites are thought to vary in location among species and may include the

base of the ear toward the mouth and the junction of the wing-membrane against the body (Hall and Richards 2000), although not all gland locations have been definitively identified (Spencer pers. comm.). Male flying foxes are not able to breed until two and a half years of age (Hall and Richards 2000). However, observations made during the present study indicate that males can begin secreting marking compounds well before they reach breeding age; the youngest male sample received was from a ten month old flying fox in Sydney. The grey-headed flying fox's neck glands are androgen-sensitive and enlarge in response to elevated hormone levels corresponding to the breeding season, beginning in January and maintained until April; this gland augmentation pattern is suspected to occur for other species and possibly other glands as well (Welbergen 2004).

Additional secretions produced in the male urogenital tract are rubbed onto the neck-ruff from the penis, where they mix with the neck gland secretions (Appendix A, Figure A3) in a process called anointing (Appendix A, Figure A4); anointing behavior has been observed in spectacled, grey-headed, black, and little red flying foxes (Spencer and Flick 1995). Anointing occurs year-round, though with a much greater frequency during mating season. Males mark their territory, an approximately 3.5 body-length segment of a branch in the roost site, during the breeding season by rubbing their shoulders and muzzle along the branch, leaving behind a marking secretion trail (Welbergen 2004). Though similar in most other respects, the little red reproductive cycle varies by six months from the other three mainland species; their peak mating time is November to December (Hall and Richards 2000).

4.2.2. Marking Compounds

Chemosignaling – passing information by means of chemical compounds that can be detected by conspecifics (members of the same species) – is a very important form of communication for most mammals (Burger 2005). Scent marking compounds are designed to be long-lasting, in order that an individual's territory or reproductive status can continue to

be displayed over days or even months (Maruani 1988). Aldehydes, alcohols, hydrocarbons, esters, fatty acids, ketones, lipids, and organic acids are some of the chemical classes that have been most commonly found in mammalian gland secretions (Stander et al. 2002, Burger 2005, Lee et al. 2007). These chemicals are generally volatile and aromatic, and evaporate relatively quickly (Cram and Hammond 1964). Compounds such as squalene and cholesterol are common in the secretions of many land-dwelling mammals and serve as fixatives to further extend the life of the volatile compounds (Wood et al. 2005, Scordato et al. 2007).

Several studies have examined Microchiroptera secretions. A study using gas chromatography in combination with mass spectrometry (GC-MS) found that the secretions on the backs of two species of long-nosed microbat males (*Leptonycteris curasoae*, *L. yerbabuenae*) included fatty acid, cholestane, and cholesterol compounds (Nassar et al. 2008). Due to the appearance of this marking patch only during times of breeding, the authors hypothesized that the patch is related to mating behavior. Compounds from the wing-sac glands of microbats have been studied most thoroughly among the Chiroptera. Brooke and Decker (1996) found a wide variety of glycolipids and nonpolar lipids beneath the wings of the fishing bat (*Noctilio leporinus*) using GC-MS.

Research on ringtailed lemur (*Lemur catta*) scent markings (Scordato et al. 2007) is especially relevant to this current study, as lemurs are actually the closest related animals to Megachiroptera (Hall and Richards 2000). Scordato et al. examined secretions from several locations and found genital secretions have a link to the time of year (breeding or non-breeding season), while other (wrist) secretions were independent. Male scrotal (genital) secretions contained primarily organic acids and esters.

Wood et al. (2005) examined four Indian and Indonesian *Pteropus* species for the compounds within the shoulder gland secretions of males. Compounds were commonly found in the classes alcohols, aldehydes, amides, carboxylic acids, esters, hydrocarbons, and

ketones. They found large differences among the species' marking compounds even at the level of chemical classes. Spencer and Flick (1995) analyzed male spectacled flying fox marking secretions using GC; in contrast to the urogenital secretions of other Australian flying fox species, which are straw-colored, the secretion of the spectacled is cranberry-red. A single major component in the secretion was found, suspected to be a long chain fatty acid, which also appears to be the cause of the distinctive smell of male *Pteropus*. The secretions were thought to originate in the prostate gland then mix with urine in the bladder before being secreted, though this could not be directly determined.

4.2.3. Australian Flying Fox Inter-species Breeding

The large distribution of the black flying fox means its territory overlaps with the spectacled in the north and the grey-headed in the south; because their breeding seasons also correspond, cases have been recorded of interbreeding between these species (Martin 1999). Although the little red's territory overlaps with all three of these other species, the six month off-set of its mating cycle prevents hybridization. Black and grey-headed flying foxes have been found to be able to interbreed and produce fertile hybrid offspring, while black and spectacled interbreeding produces offspring that resemble only the spectacled parent (Webb and Tidemann 1995). No cases of spectacled and grey-headed flying fox interbreeding have been recorded, as their home ranges do not connect.

4.3. Rationale and Aims

In order to fully appreciate the influence of chemical signaling on community and ecosystem processes, a thorough knowledge of the mechanisms of chemical information conveyance from the gene to the individual is needed (Takken and Dicke 2006). Therefore, the highly multidisciplinary approach of modern chemical ecology is likely to make an important contribution to biology in the 21st century. The nature of chemical information transfer mandates further understanding of interactions based upon knowledge of the

chemicals involved, which can range from highly volatile to non-volatile compounds. Organisms can produce a vast diversity of chemicals, often in minute amounts. Modern analytical technology allows for their identification by standard methodology, usually GC-MS or high pressure liquid chromatography (HPLC).

Flying fox populations are currently experiencing drastic declines from habitat loss, culling by fruit farmers, tick paralysis, electrocution on powerlines, and becoming trapped on barbed wires fences, combined with a slow population growth rate (Fox et al. 2008). Consequently, the spectacled and grey-headed flying foxes are currently considered vulnerable (Department of Sustainability and the Environment 2008). Furthermore, bats play important ecological roles across the globe as either dispersers and pollinators or controllers of insect populations. A better understanding of *Pteropus* communication can lend insights into their social and reproductive behaviors, and thereby assist in the conservation of flying foxes and other bat species.

Although most Chiroptera, including megabats, are social animals that roost and raise their young in colonies, only a very few studies have examined communication (vocal and non-vocal) in bats. Little is known about communication between group members in bat societies (Dechmann and Kamran 2005); minimal research has been conducted on Microchiroptera, and even less on Megachiroptera. Therefore, almost nothing has been systematically studied about megabat marking secretions. To begin resolving this Megachiroptera knowledge gap, this study aims to examine three species of flying foxes in Australia for similarities and differences in the neck-ruff marking secretions, which are composed of a mix of contributions from the urogenital track and neck glands. Further, this study aims to see if the secretions of spectacled, grey-headed, and black flying foxes are relatively similar, given that interbreeding can occur, but still distinct, given that they are considered different species.

5. Methodology

5.1. Sample Collection and Preparation

The study was conducted at the Cape Tribulation Tropical Research Station, located in the Daintree Rainforest of North Queensland, Australia. This station houses four adult male and two adult female, non-releasable spectacled flying foxes. To collect samples from these bats, a small piece of clean paper tissue was rubbed vigorously against the neck of the animal until the tissue was thoroughly wet with marking secretion; the tissue was then sealed into a clean plastic bag. Outside facilities from southern Queensland to southern New South Wales, with colonies of grey-headed and black flying foxes, were contacted to send in samples. Personnel at the offsite facilities were instructed to collect secretion specimens in the same manner as the spectacled flying fox samples. Flying fox ages of sampled animals ranged from ten months to twenty years. Comparison samples from a male spectacled flying fox of overnight-fermented neck secretion and fresh, newly anointed secretion were run; no obvious differences occurred in content or relative amounts of components, so the time of anointing prior to sample collection was not a variable examined.

Collected samples were prepared for HPLC runs by solvent extraction, a technique used when the compounds being tested are soluble in organic solvents – methanol in this case. A 0.25 centimeter square piece of paper tissue, saturated with the sample, was removed from the main collection tissue and placed into a small vial. For most samples used in pre-testing, a concentrated solution was made by adding 0.5 mL of methanol and then diluted 1:20 by placing 10 μL of concentrated sample and 0.2 mL of methanol into a new vial. For samples used in the ultimate pre-testing stages and in final analysis, 3.0 mL of methanol was added directly to the vial with the 0.25 cm piece of sample tissue. Original collection tissues and concentrated sample vials were stored in a freezer (-8°C). All other samples were stored at room temperature (30°C).

5.2. HPLC

5.2.1. Background

Secretion samples were analyzed with HPLC by adsorption chromatography. A sample was injected into the HPLC column where a layer of sample molecules (the solute) coated the surface of silica (the adsorbent) that filled the inside of the column. The sample compounds became adsorbed onto the column while the eluting solution (running solution), flowing continuously through the column, caused the sample components to differentially separate. Separation is based on the varying degrees of bonding to the column, depending on the components' chemical properties. All runs were conducted isocratically (uniform composition of the running solution), so no elutant gradients were used. As the compounds left the column, they were detected as changes in UV absorbance by a spectrophotometer. The resulting peaks indicated the sample components that show absorbance in the UV spectrum; more than one compound may be represented within a single peak, depending on the resolution (peak separation) created by the choice of column type, running solution, and other running settings.

5.2.2. Settings

The HPLC (ISCO Inc., Model 2350) had an elutant flow rate of 1.00 mL/min and an operating pressure ranging between 2000-3000 psi. Injections were made using a gas-tight syringe, capable of injecting 1.0 to 10.0 μL volumes. The Liquid Chromatography UV Spectrophotometer (Waters-Millipore, Lambda-Max Model 481) was set to measure absorbance at a detection wavelength of 254 nm, which is commonly used for organic substances that often strongly absorb (Skoog and Leary 1992). Data was recorded on a chart recorder (LKB Bromma, 2210 2-Channel Recorder) that plotted the peaks on paper as a function of time. Settings for the recorder were a paper speed of 0.2 mm/sec, 0.5 mm/sec, 10 mm/sec, or 10 mm/min (depending on other parameters) and a sensitivity of 20 mV or 50mV

(depending on how concentrated the samples were).

Prior to running samples each day, the performance of each column was tested and compared to a standard that had been made upon the purchase of each column, using the same running conditions and test mix solution. The test mix used was the Supleco HPLC Isocratic Systems Diagnostics Mix (Sigma-Aldrich, 48270-U) that contained four compounds (ethyl, methyl, propyl, and butyl 4-hydrobenzoate), each generating a distinct peak. Eight microliters of running solution were drawn up into the syringe, followed by 2 μL of test mix for each injection; the initial addition of eluting solution provided a "wash-out" of the injector port and tubing, to prevent carry-over of components into later runs.

5.2.3. Pre-testing

Before beginning the actual sample analysis, the ideal running conditions had to be found that maximally separated the components of the secretions. This preparation process occurred over 25 days and involved testing various running conditions – four different columns (SunFire C18, 5 μm silica coating, 4.6 mm inner diameter x 150 mm length, Waters; μBondapax C18, 5 μm, 3.9 mm x 300 mm, Waters-Millipore; Zorbax SB-C18, 5μm, 4.6 mm x 150 mm, Agilent; Luna C8(2), 5 μm, 4.6 mm x 150 mm, Phenomenex) and 11 running solutions (100% methanol; 90% methanol:10% water; 80% methanol:20% water; 70% methanol:30% water; 60% methanol:40% water; 50% methanol:50% water; 10% methanol:90% water; 100% water; 60% acetonitrile:40% water; 40% 50 mM potassium di-hydrogen phosphate:60% methanol; 70% ethanol:30% water) were examined. Running solutions were degassed prior to use, to prevent introducing air bubbles into the column and causing false peaks to appear on the readout. The conditions yielding the best peak resolution were the SunFire C18 column with 60% methanol:40% water, and this configuration was subsequently used in all sample runs. Additionally, running solution flow rates of 1.00 mL/minute and 0.250 mL/minute were both examined; the former was found to be more

efficient while yielding equivalent resolution and was therefore used throughout most of the experiment.

The secretion samples used during this testing process to find the best running conditions were dissolved in methanol solvent and were collected from two spectacled ("Jasper" and "Pushkin"), two grey-headed ("Koda" and "Boris"), and one black ("Bear") flying foxes. Two solvents had been experimented with for getting the secretions into solution: the first, 60% methanol:40% water, as a solvent was found to result in hydrolysis; therefore, pure methanol was chosen for use throughout the study. Most injections during pre-testing involved a 5 μL running solution "wash" drawn up prior to the 5 μL of sample; injections used in the ultimate pre-testing stages and in final analysis involved an 8 μL running solution "wash" drawn up prior to the 2 μL of sample.

5.3. Sample Analysis

The running conditions determined by the pre-testing included a SunFire C18 column, methanol solvent, 60% methanol:40% water eluting solution, 1.00 mL/minute flow rate, joint-wash injection including 8 μL running solution and 2 μL sample, 20 mV recorder sensitivity, and 0.5 mm/sec paper speed; these were held constant for all runs. Three types of blanks were first injected: 10 μL 60% methanol:40% water, 8 μL 60% methanol:40% water with 2 μL pure methanol, and 8 μL 60% methanol:40% water with 2 μL sample blank (0.25 cm of clean tissue in 1.0 mL methanol). The species and individual for each sample were also recorded for every run. Species and genders of flying foxes analyzed included spectacled males, grey-headed males, black males, and spectacled females. Each individual sample was tested successively; once all runs had been completed, a repeat was conducted for all blanks and samples. All final sample analyses were completed over the course of one day to reduce the number of possible confounds.

5.4. Data Analysis

Peak distances were calculated by measuring the distances between the start of a run and the very top of a peak, for all major (greater than 5 mm above baseline) sample peaks; measurements were rounded to the nearest 0.25 mm. For less distinct, but still discernable, peaks, a ruler was used to locate the point at which the slope changed, indicating the meeting of two major compounds. Differences in peak height indicate variation in compound absorbance, while peak widths indicate the volume of substance. Values are presented as the mean ± standard deviation unless otherwise indicated.

6. Results

The secretions collected from the neck-ruffs of spectacled males (N = 4), grey-headed males (N = 4), black males (N = 3), and spectacled females (N = 2) were analyzed in this study. Among these species, a total of six unique, major peaks were discernable and have been arbitrarily labeled Components A-F (Table 1, Figure 2). Not all species and sexes possessed all of these components; only black males had Component D, and spectacled females did not show Component F (Table 1, Figure 3). Little variation was present between groups for the peak distances (Figure 3, Figure 4), indicating that the same compounds were present among most groups. Differences did occur between groups in the relative amounts of many secretion components (Figure 4).

	Mean Peak Distances (mm) ± SD of Major Components					
Species/Sex	A	B	C	D	E	F
Spectacled Male	49.2 ± 0.2	57.6 ± 1.1	60.3 ± 1.3	-	70.7 ± 1.7	77.6 ± 2.8
Grey-headed Male	48.9 ± 0.3	57.6 ± 0.5	60.7 ± 0.8	-	71.6 ± 1.1	76.8 ± 1.6
Black Male	49.0 ± 0.2	58.4 ± 0.7	61.8 ± 0.5	64.2 ± 0.6	71.8 ± 1.0	77.7 ± 2.7
Spectacled Female	48.7 ± 0.2	58.2 ± 0.6	61.1 ± 0.7	-	72.0 ± 1.1	-
All	49.0 ± 0.3	57.9 ± 0.8	60.9 ± 1.0	64.2 ± 0.6	71.4 ± 1.3	77.3 ± 2.3

Table 1: Mean Peak Distances of Major Components by Species and Sex

Among the discernable HPLC peaks, spectacled and grey-headed males possessed Components A, B C, E, and F; black males had all components; and spectacled females had Components A, B, C, and E.

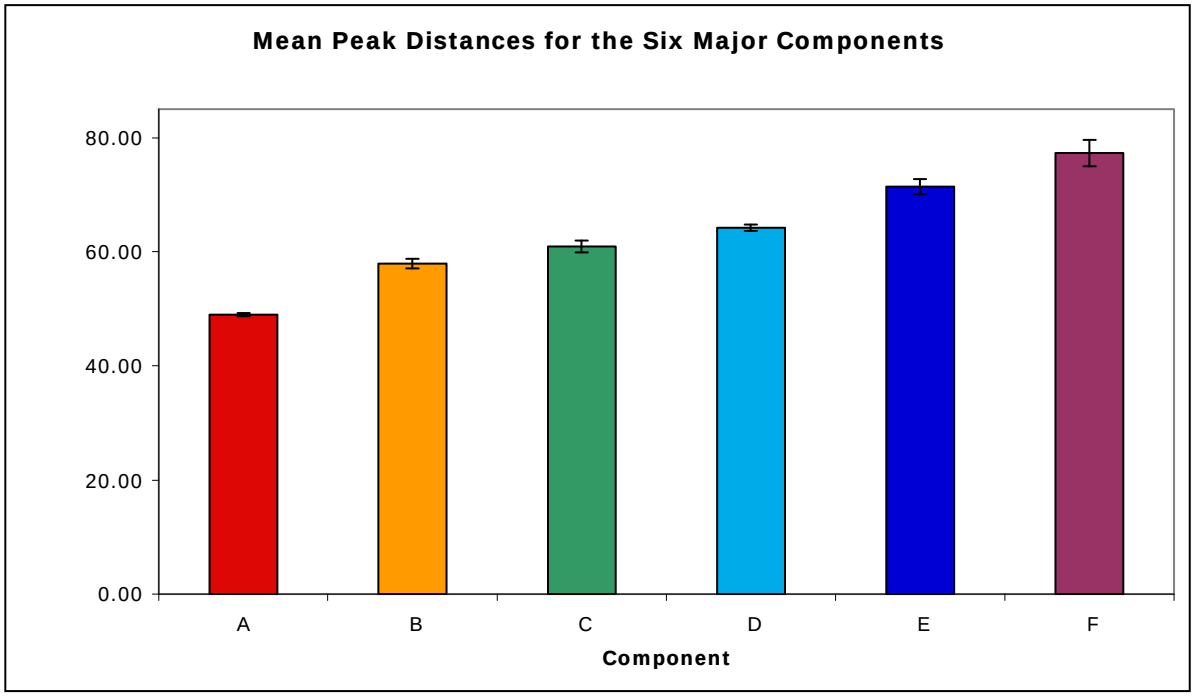

Figure 2: Mean Peak Distances of Major Components

Six major peaks (Components A-F) were able to be differentiated from the HPLC readouts. All peaks are considered distinct from each other, as none of the standard deviations overlap between compounds. Error bars are given as ± one standard deviation.

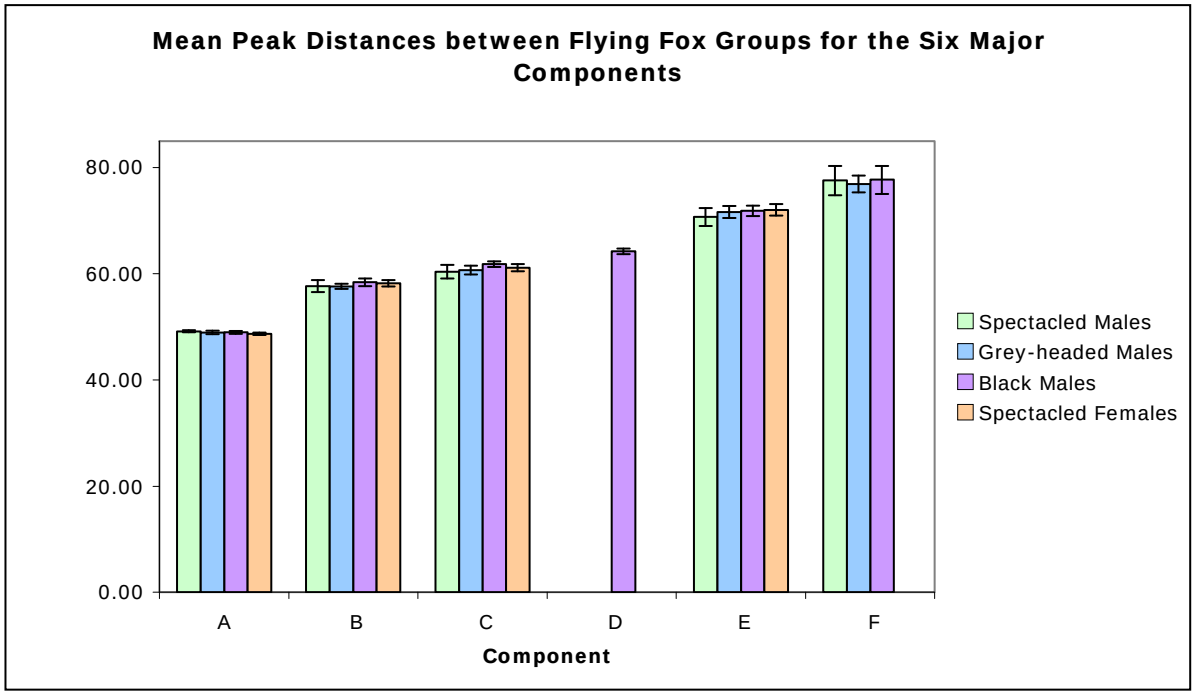

Figure 3: Mean Peak Distances Among Flying Fox Groups

Little variation is present between the four flying fox groups for each of the major components, which indicates that the peaks are the same between the species and sexes examined. Spectacled males (N = 4) and grey-headed males (N = 4) possessed compounds A, B C, E, and F; black males (N = 3) had all components; and spectacled females (N = 2) had components A, B, C, and E. Error bars are given as ± one standard deviation.

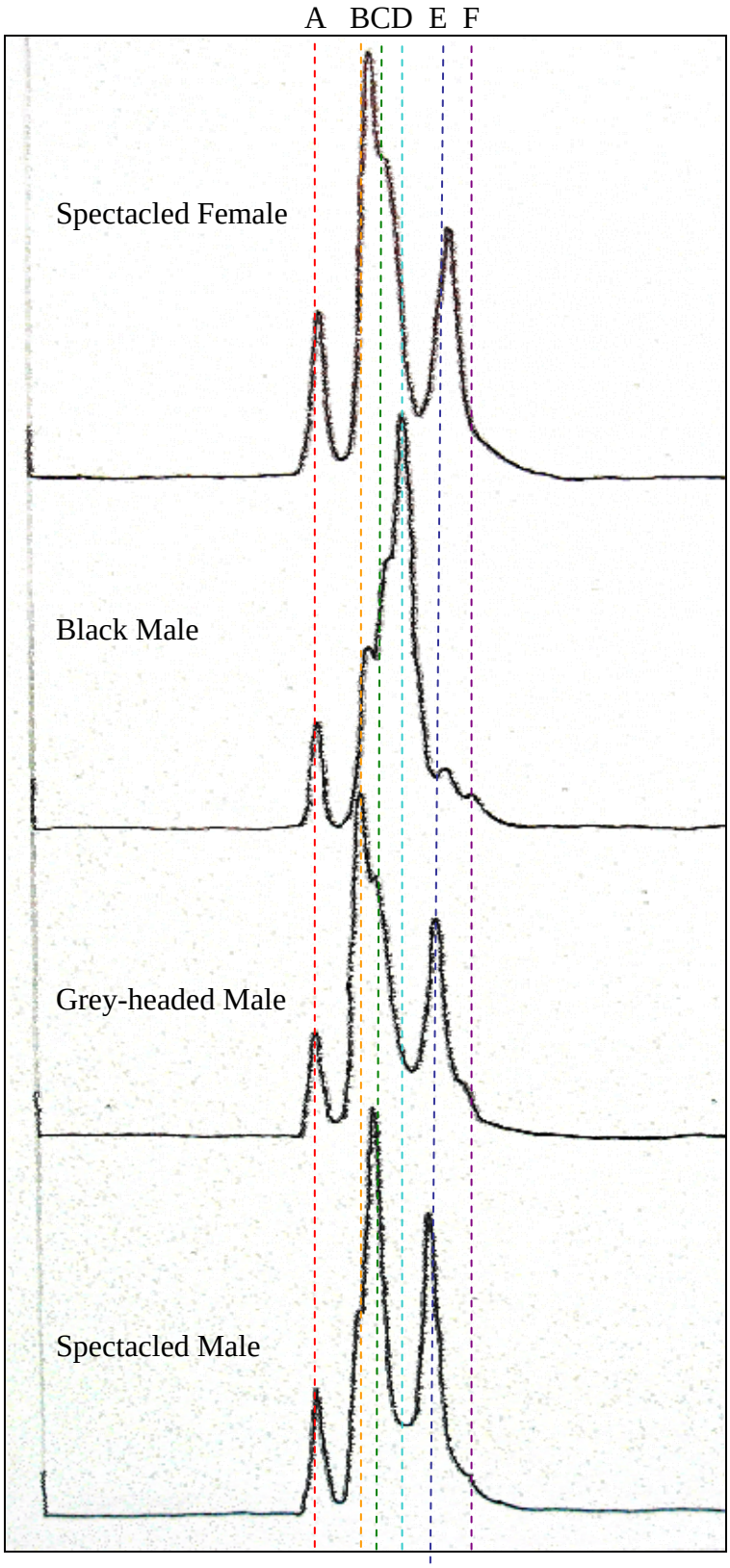

Figure 4: Stacked HPLC Plots from Example Individuals

This figure illustrates the six major compound peaks for the spectacled male, grey-headed male, black male, and spectacled female flying foxes, using representative individuals from the first round of result runs. The small lines on the far left indicate the injection point (start of each run). The colored lines show the relationship between the major compound peaks among the four flying fox sample groups. Component A is labeled with a red line, B is orange, C is green, D is pale blue, E is dark blue, and F is purple. Component D is present only for black males; Component F is absent for spectacled females.

7. Discussion

7.1. Interpretation of Results

Data indicated that the males possessed a unique neck-urogenital secretion chemical profile for each species examined, though they did contain many similar components (Figure 3, Figure 4). Spectacled and grey-headed male flying foxes appeared to differ only in the relative amounts of the major components (Figure 4), while black males had one more major component than either of the other two species (Figure 3). Female secretions contained one fewer major component than the secretions of the males, though still sharing four of the five major compounds found for the males of all three species (Figure 3).

It is important to note that the definition of "major component" here is determined by the resolution ability of the equipment used during the present study. It is possible that more than one actual component is contained within one visible peak, but the analytical conditions used were not able to adequately separate these components; most other minor components would be unable to be detected. Therefore, the observed differences among the species are "indicative," rather than definitive.

Definite differences were observed between individuals of the same species; though the individuals' secretions retained the overall pattern of relative component amounts for the species, there were slight variations within these general trends (Appendix C, Figures C4-C16). Lemurs have also been observed to have individual scent profiles in previous research studies (Scordato et al. 2007).

The collection of female neck secretions aimed to differentiate which male secretion components originated from the neck gland and which originated in the male urogenital tract, since females are anatomically incapable of producing urogenital secretions. It was assumed that the female secretion components would reveal which compounds were neck gland related, and from there, extrapolations could be made to determine what the urogenital

secretions must be. However, because male flying foxes often rub against females, it is not possible to definitively determine whether the samples collected from the female spectacled flying foxes contained only the female's own neck gland secretion or whether samples had been contaminated by interaction with a male, and therefore the male's combined contribution of urogenital and neck secretions. Based on the volume of sample collected from one spectacled female ("Sunshine"), it is likely she received at least some male neck secretion through physical contact with a male. To resolve the problem of differentiating male and female secretions, future studies should examine the secretions of females without the possibility of physical contact with males. The females should, however, remain in close proximity to the males – having a simple separation such as a wire divider between two enclosures – to prevent any glandular changes associated with lack of male pheromones or presence. Additionally, the components of the neck gland secretions of males and females are likely to differ, so it should not be assumed that contents not found in female neck gland secretions are from the male urogenital track.

Age differences in glandular secretions have been suspected in some species, such as weasels and deer, while other species, such as mice, do not show any age-related variation in secretions (Burger 2005). The spectacled males examined in the present study were all fifteen to sixteen years of age, while most of the grey-headed males were less than three years old. Additionally, both female spectacled flying foxes were quite old – eighteen to twenty years – which may have caused them to secrete substances of different composition or concentration than younger females would. The twenty year old ("Seraphina") had almost no neck secretion at all, which may to be due to her age; most flying foxes in captivity do not live much more than twenty years (Fox et al. 2008). Therefore, differences in secretions due to age may have occurred in this study, but as no mammal studies have definitely shown differences in the composition of glandular secretions due to age, no research precedent exists

for this interpretation.

7.1.1. Possible Secretion Compound Identities

A major previous study (Spencer and Flick 1995) was a precursor to this current investigation. The study began to investigate the composition of spectacled flying fox glandular secretions. Their experiment was based on the assumption that fatty acids were the principal component of the secretions. In the 1995 study, a procedure for breaking down fatty acids, to produce a derivative of the major secretion component, was carried out. The product of this methylation derivitization was then analyzed using gas chromatography.

Spencer and Flick's procedure is now thought to have not worked as anticipated (Spencer pers. comm.). One indication of unexpected results was due to the derivative's properties. Normally, a chemical is altered during derivitization, and the derivative lacks the parent compound's features. In the 1995 study, as the substance that produced the largest chromatographic peak emerged from the column (Appendix B, Figure B1), the strong smell of the spectacled flying fox was distinguishable (with the detector temporarily disconnected); this indicated that this component was still active and probably was present in unmodified form. The derivitization process had been designed for fatty acid materials, so the compound is now thought to not be a fatty acid. Additionally, fatty acids are not soluble in water, and the compound being examined has been determined to be water-soluble. This further supports the hypothesis that the substance is not a fatty acid, but belongs to an entirely different chemical category.

The compounds are now suspected to be alcohols, hydrocarbons, esters, or ketones based on the findings of Wood et al. (2005). The aforementioned chemical classes are the groups that made up the greatest portions of the neck secretions for at least one of the four flying fox species that Wood et al. examined. The other categories – which contributed only minor components – included aldehydes and amines; though fatty acids (carboxylic acids)

were also major components, they were not considered here due to the previous findings of Spencer and Flick (1995). All of these major chemical groups can be biologically produced by organisms and usually contain many odiferous members (Cram and Hammond 1964); these results are consistent with the findings of Spencer and Flick (1995) that the major secretion component carries the flying fox odor.

The GC trace (Appendix B, Figure B1, Spencer and Flick 1995) additionally shows at least one of the major components to be of low molecular weight, since it was eluted as one of the first compounds in the secretion and lighter molecules progress through the column faster. Because the secretion compound containing the odor is also separated early in the HPLC, further support is provided that the odor-containing compound is of low molecular weight.

The methanol blank and clean tissue blank runs both included a peak with a distance that was not significantly different from Component A (Appendix C, Figure C2-C3). In the blank runs, the peak must be caused by the additional methanol present (relative to the running solution); it can further be inferred that the samples contained a component that may be similar in structure to methanol – an alcohol – due to the similarity in peak distances. However, the sample Peak A's cannot be entirely caused by the methanol solvent, due to the much greater size of these sample peaks in comparison to the blanks, though methanol is likely to be a masked peak within Component A if the secretions themselves do not contain methanol.

7.2. Limitations and Future Studies

Measurements of peak distances were made by hand, rather than being automatically calculated by a computer; therefore, slightly imprecise readings may have arisen from the hand calculations. Additionally, injections were performed by hand, rather than being auto-

injected by the machine; the manual injections create another possibility for small variations in the results, but should have relatively little effect on the timing of peak distances.

Though many different running conditions were tested, given the restricted choice of columns and eluting solutions available, the best possible combination may not have been found. So, better resolution may be possible using other columns or elutants after further evaluation. Additionally, in future studies, knowing the chemical class of these compounds would assist in picking running solutions that would maximize separation based on the chemical properties of the compound.

As the tool available at Cape Tribulation Research Station, HPLC was an effective method to initially access the similarities in marking secretions among *Pteropus* species. However, using GC to get better separation resolution – especially for compounds making up only a small percentage of the total secretion – and MS to find the exact identities of compounds would assist greatly in learning more about flying fox secretions. More detailed resolution obtained from GC-MS or another method could serve as a useful tool for looking at relationships, as well as allowing better comparison with other existing studies. Previous studies (Spencer and Flick 1995) indicate that GC has the potential to yield far better resolution of the compounds present in flying fox secretion samples (Appendix B, Figure B1).

An interesting next preliminary step would be to determine which components found in the secretions originated from the urogenital track and which came from the neck glands. To determine this, some urogenital secretion could be intercepted, before coming into contact with the neck-ruff, and then analyzed. This analysis would reveal the components not found in the urogenital secretions, which could then be assumed to originate from the neck glands.

Studies have found multiple factors that could generate differences in secretion composition: analysis by gender, by seasonality, between camps or groups, and between

individuals. Consequently, examining the role of these factors in flying fox secretions could yield interesting and more in depth findings, once appropriate analytical system conditions have been determined to permit exploration in these areas.

Running analyses of the female neck secretions for the other two flying fox species – grey-headed and blacks – in addition to the spectacleds studied here and then comparing the new female secretions to male secretions could reveal two interesting possibilities. Either there would be more similarities between species within genders, in which case all males would be more similar to each other than they are to any female and vice versa (as found by Brooke and Decker 1996), or more similarities within species will occur regardless of gender, in which case males and females of the same species would be more similar to each other than to any member of another species. Data from spectacled flying foxes revealed that male secretions are more similar to black and grey-headed male secretions than to female secretions, due to differences in total component numbers (Figure 3). Further studies could reveal whether this pattern holds true for the other two species as well.

Studies of other mammals reveal another finding related to gender; though male and female pandas (*Ailuropoda melanoleuca*) were found to have similar scent mark and genital secretion compositions, the concentrations of the components varied greatly between sexes (Hagey and MacDonald 2003). Male and female spectacled flying foxes may have a similar trend (Figure 4); though this has not yet been determined due to the possible contamination issue, it could be clarified by clean samples and improved resolution.

Differences have also been observed in secretions between seasons, primarily defined by mating or non-mating times of year. Variation in lemur genital secretion contents and concentrations was found during and outside of the breeding season (Scordato et al. 2007). Megabats should be similarly examined cross-seasonally to see if the secretion composition changes or if individual components vary in amount.

Brooke and Decker (1996) found that, in some cases, secretions of male fishing microbats (*Noctilio leporinus*) within a single camp were more similar to each other than to outside males or to any females; similarly, Bloss et al. (2002) found that big brown microbats (*Eptesicus fuscus*) could differentiate between colony mates and outsiders based solely on secretion scents. Further studies to examine camp differences in *Pteropus*, using more sensitive techniques like GS-MS to pick up the minute variations, would be another area for future research to improve our knowledge of Megachiropteran communication. Individual differences, as observed in this study, would also be more discernable using GC in later analyses.

Once the composition of the spectacled, grey-headed, and black flying fox secretions has been determined, it would be interesting to compare them to little red flying foxes, which cannot interbreed with any of the three examined species; to other Australian Megachiroptera, like tube-nosed fruit bats (*Nyctimene* spp.) and blossom bats (*Syconycteris* and *Macroglossus* spp.); or to species outside of Australia, such as the four *Pteropus* species from India and Indonesia examined by Wood et al. (2005) to see whether any components are shared. Additionally, more direct comparison with lemurs, the closest living relative of the Megachiroptera, may offer insights into their shared evolutionary heritage.

7.3. Conclusions

This study found that the secretions of spectacled, grey-headed, and black flying foxes, while still distinct from each other, are similar in components. This finding is consistent with known flying fox behavior and classification, in that interbreeding is possible and therefore indicates that the species must still be closely related but are different enough to be considered three different species.

The hybridization of black with grey-headed and spectacled flying foxes leads to further questions regarding what similarities these different species have that both allow them to recognize each other as mates and to actually produce young. It is possible that similarities

in marking secretions provide chemical recognition of potential mates, which might be more important than the small external physical differences between species.

Each species of flying fox examined in this study had a significantly different characteristic odor, found primarily on the males. The variation in odors appears to stem from one of the major components of the neck-urogenital secretions, as samples carry this distinctive scent as well. The urogenital secretions are strongly suspected to originate from an internal gland or organ and then mix with urine in the bladder (urine-bathing) before being released (Spencer pers. comm.). This process is hypothesized because 1) post-mortem bladders of male spectacled flying foxes were found to contain the cranberry colored marking fluid and 2) the straw-colored marking secretions of the other three species are thought to be due to mixing a yellow or clear marking compound with urine (Spencer and Flick 1995). The gland of origin is hypothesized to be the prostate, as it is the only gland that appears large enough to produce the volume of secretion the males excrete; however, the urogenital secretion source has not yet been determined and remains an interesting topic to explore.

With the rise in technology – especially HPLC, GC, and MS – secretions have begun to be studied in many organisms over the past twenty years. However, chemical ecology is still a newly budding field, with many areas and species still to be examined. Although the totality of chemical communication is beyond the scope of this project, studying glandular secretions could be extended into the areas of mate selection, bond or territorial maintenance, evolutionary aspects of scent communication, other functions of signaling behavior, and the overall social health of a colony through recognition or promotion of cooperative behaviors. Knowledge gained through secretion studies could also be adapted to conservations efforts, including the sharing of territories by different *Pteropus* groups as flying fox habitat continues to be reduced and arguments for the maintenance of appropriate amounts of habitat due to conflicting needs of the different species.

8. Appendices

8.1. Appendix A: Male Anatomy and Behavior

Figure A 1: Spectacled Flying Fox Testes

Figure A 2: Spectacled Flying Fox Penis

The outer anatomy of a male spectacled flying fox at rest with penis retracted into his body cavity (Figure 2) and with penis exposed during an erection (Figure 3).

Figure A 3: Spectacled Flying Fox Marking Secretion

The neck-shoulder gland secretions combined with urogenital secretions on the neck of the spectacled flying fox gives the fur a cranberry-red coloration. The secretions for black and grey-headed flying foxes are straw-colored.

Figure A 4: Spectacled Flying Fox Anointing

A male spectacled flying fox in the process of anointing, during which he achieves an erection and then proceeds to wipe the tip of the penis – which is releasing the urogenital secretion – on either side of his neck where it combines with the neck secretion.

8.2. Appendix B: Gas Chromatography Trace, Spencer and Flick (1995)

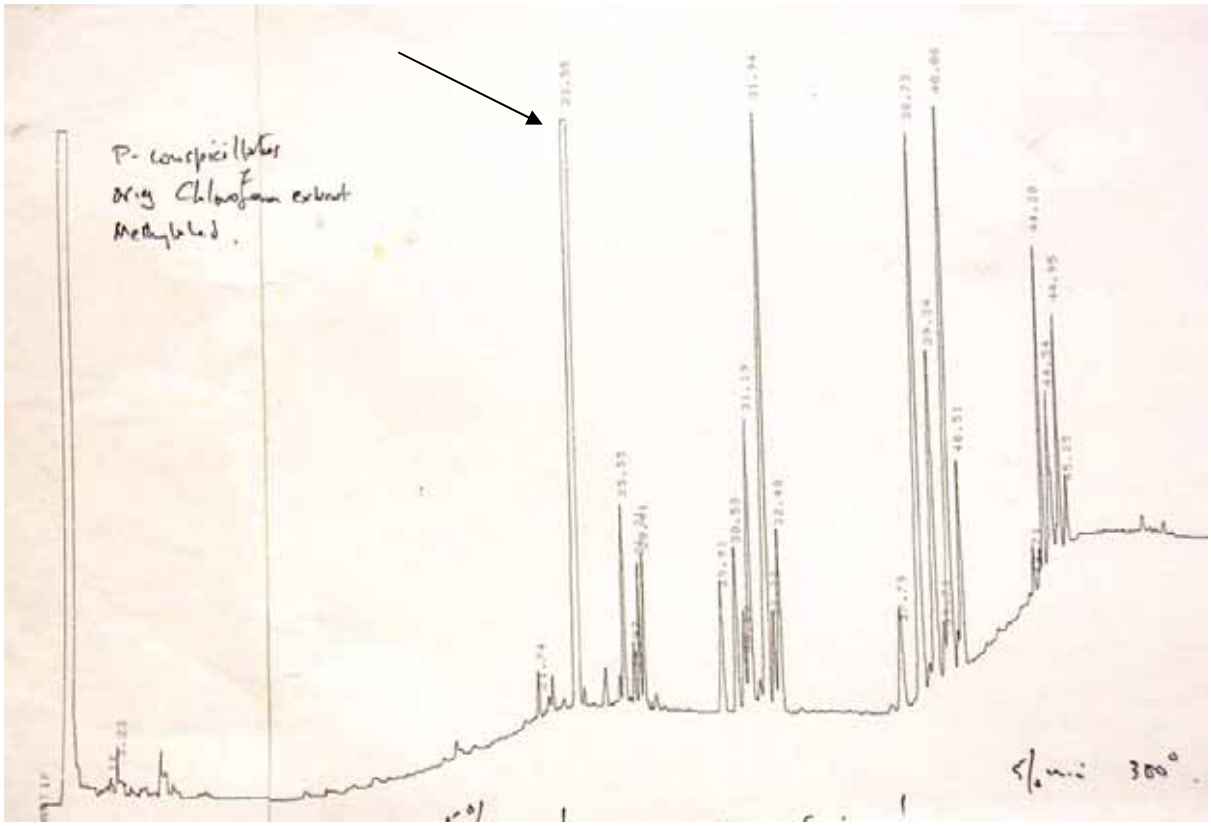

Figure B 1: GC Trace from Spencer and Flick (1995)

This readout shows the GC results from a run of male spectacled flying fox neck-urogenital secretion after the fatty acid derivitization procedure had been carried out. The highest peak is at 23.55 seconds (see arrow), and had such a great height (which indicates a large percentage of the secretion content) that it exceeded the top of the page and instead appears as a flat line for the peak top. The area under this peak is about five times greater than the next largest peak, clearly indicating the 23.55-second peak is the major compound. The numerous smaller peaks are minor components of the secretion.

8.3. Appendix C: HPLC Plots

HPLC plots are given for the first round of final testing. Less noticeable peaks are marked with an arrow. Peaks less than 5 mm above baseline were not included. The small lines on the far left indicate the injection point, or the start, of each run. The paper grid sizes are 10 mm for each horizontal segment and 2 mm for each small vertical unit (20 mm for the larger vertical grid lines).

8.3.1. Blanks

The component of each "blank" injection is given in parentheses.

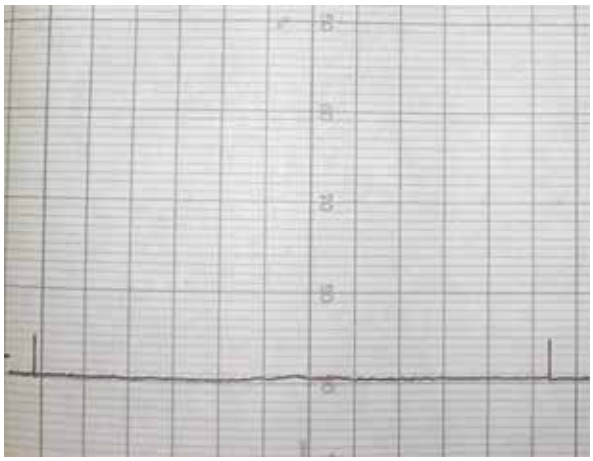

Figure C 1: Blank 1 (Running Solution)

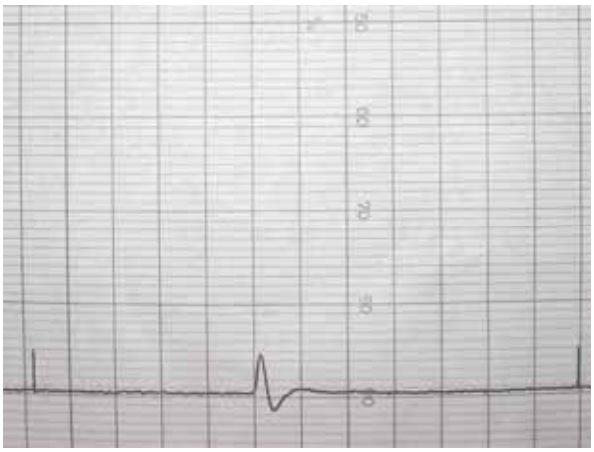

Figure C 3: Blank 3 (Clean Tissue)

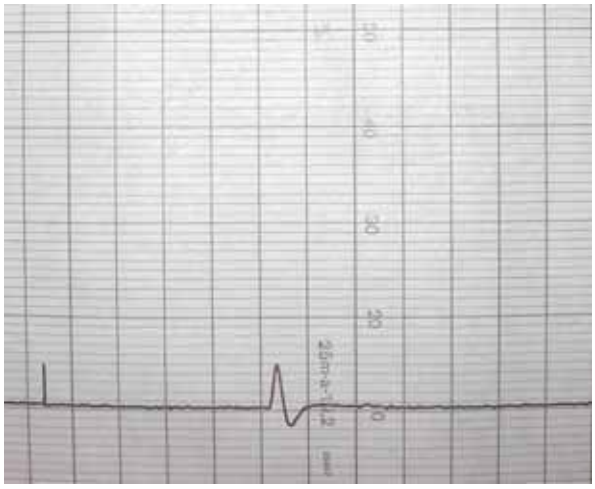

Figure C 2: Blank 2 (Methanol)

8.3.2. Spectacled Males

Each of the four male spectacled flying foxes showed 5 major peaks – Components A, B, C, E, and F.

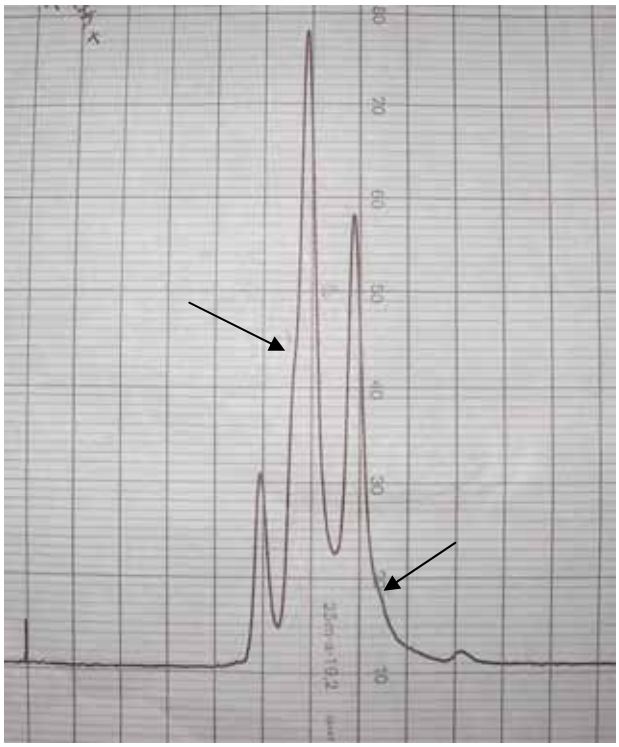

Figure C 4: Spectacled Male 1 ("Jasper")

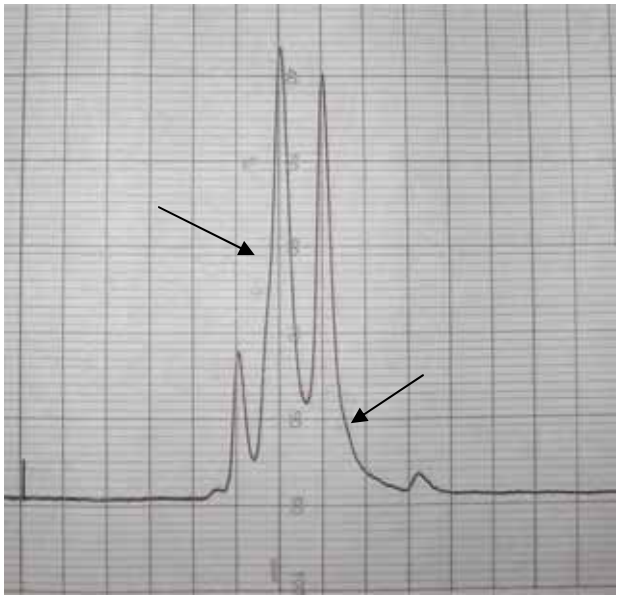

Figure C 5: Spectacled Male 2 ("Pushkin")

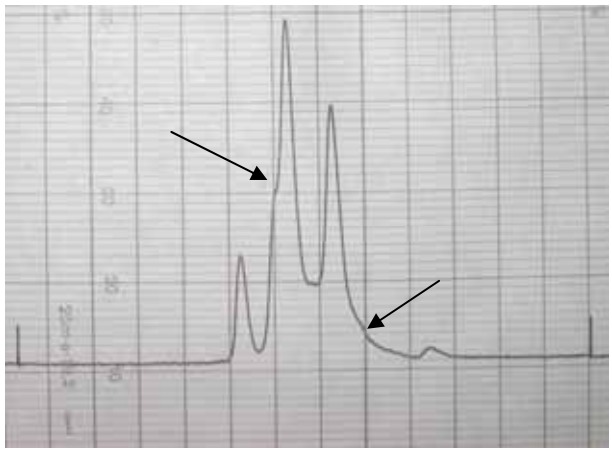

Figure C 6: Spectacled Male 3 ("Rex")

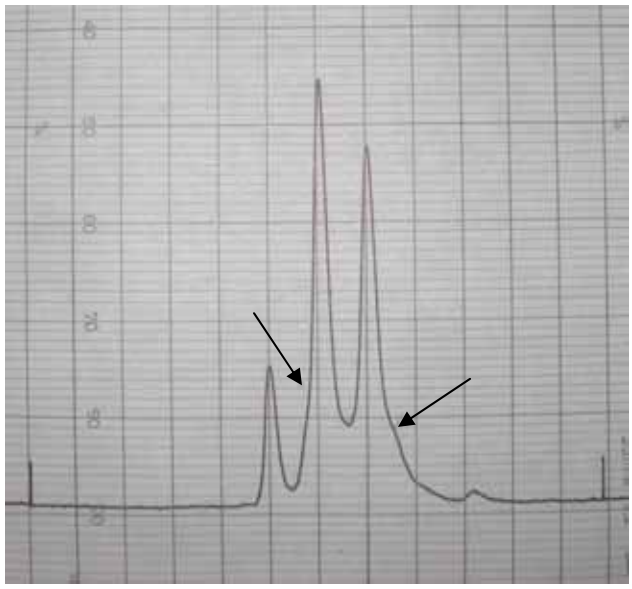

Figure C 7: Spectacled Male 4 ("Old Boy")

8.3.3. Grey-headed Males

Each of the four male grey-headed flying foxes showed 5 major peaks – Components A, B, C, E, and F.

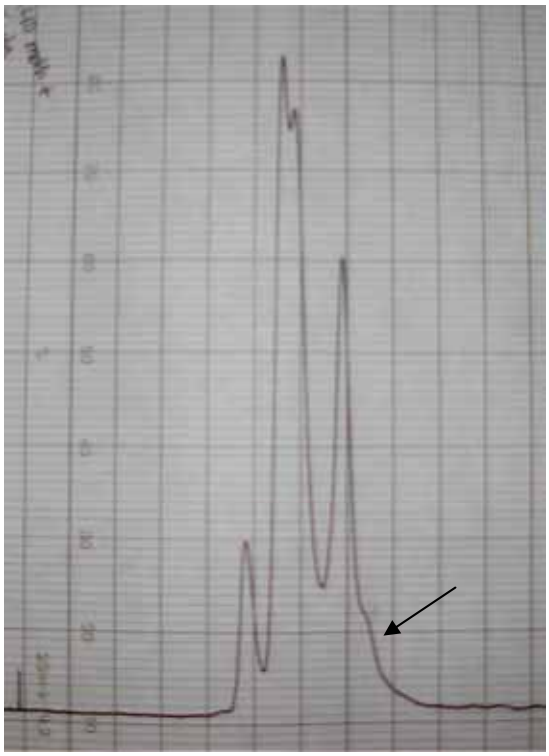

Figure C 8: Grey-headed Male 1 (“Koda”)

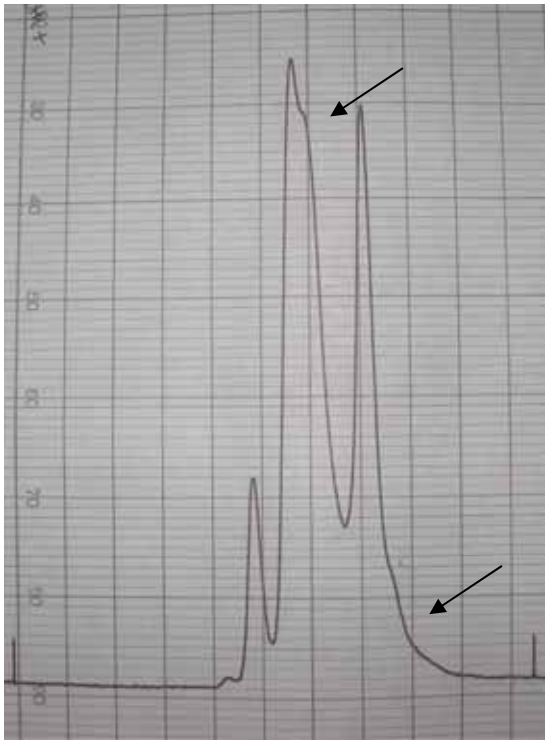

Figure C 9: Grey-headed Male 2 (“Boris”)

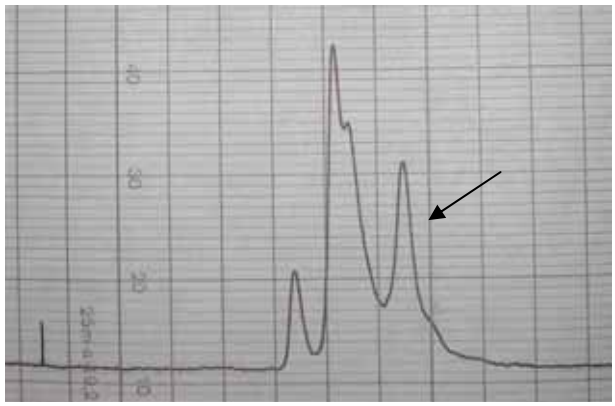

Figure C 10: Grey-headed Male 3 (“Reggie”)

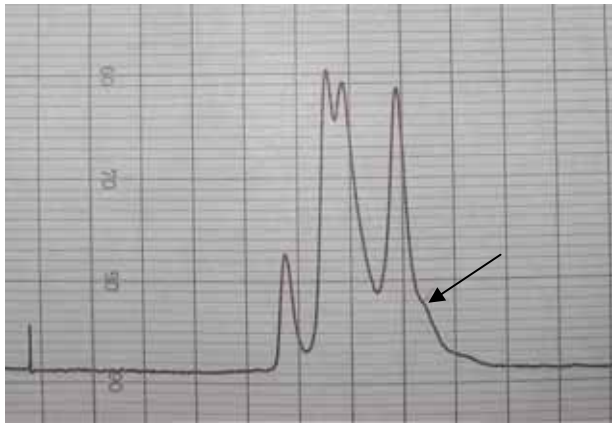

Figure C 11: Grey-headed Male 4 (“Alfi”)

8.3.4. Black Males

Each of the three male black flying foxes showed 6 major peaks – Components A, B, C, D, E, and F.

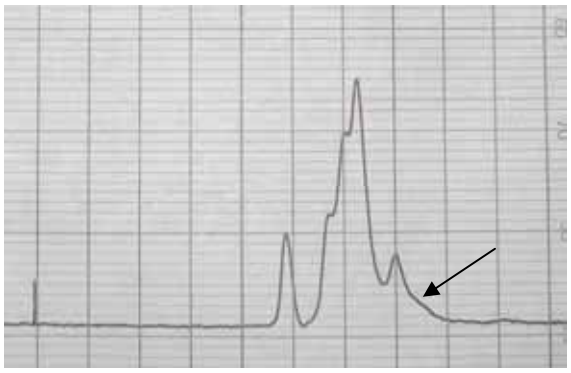

Figure C 12: Black Male 1 ("Alli")

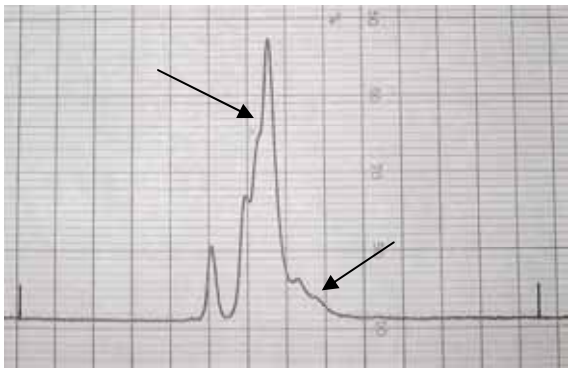

Figure C 14: Black Male 3 (“Cat”)

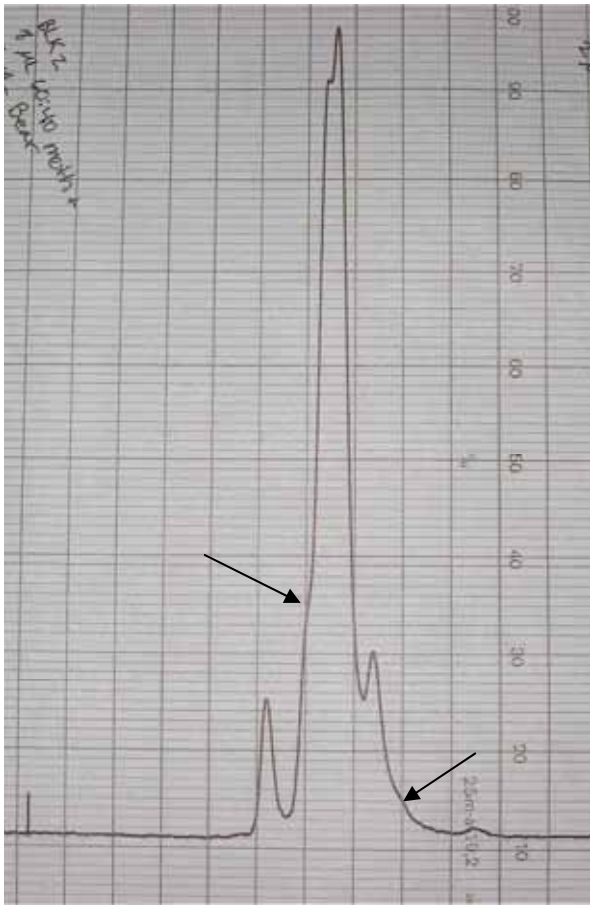

Figure C 13: Black Male 2 (“Bear”)

8.3.5. Spectacled Females

Both of the female spectacled flying foxes showed 4 major peaks – Components A, B, C, and E.

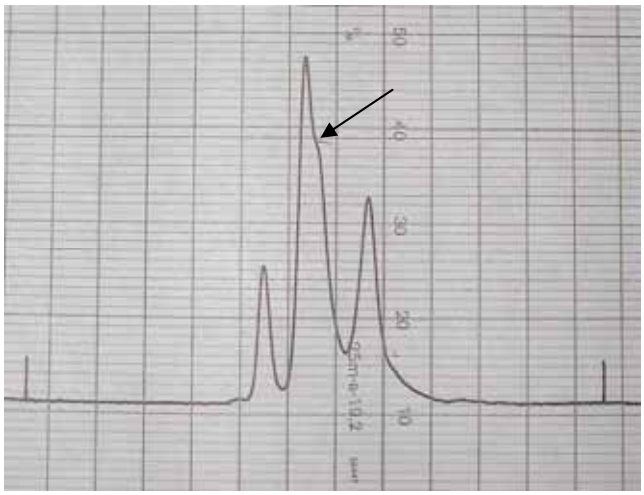

Figure C 15: Spectacled Female 1 ("Sunshine")

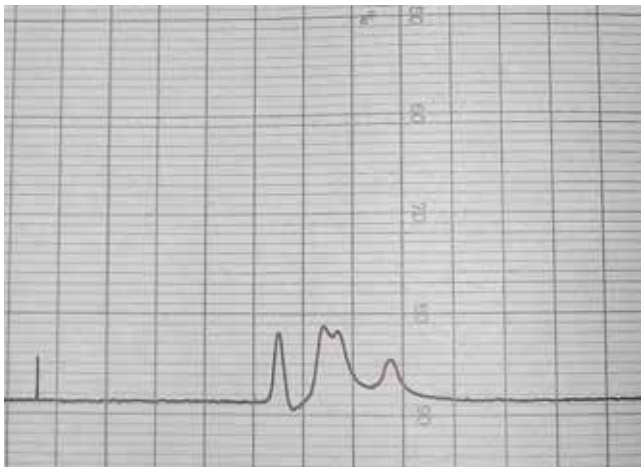

Figure C 16: Spectacled Female 2 ("Seraphina")

9. References

9.1. Literature Cited

Bloss J, Acree TE, Bloss JM, Hood WR, Kunz TH. 2002. Potential Use of Chemical Cues for Colony-Mate Recognition in the Big Brown Bat, *Eptesicus fuscus*. Journal of Chemical Ecology 28(4): 819-834.

Burger BV. 2005. Mammalian Semiochemicals. Topics in Current Chemistry 240: 231-278.

Brooke AP, Decker DM. 1996. Lipid Compounds in the Secretions of Fishing Bat, *Noctilio leporinus* (Chiroptera: Noctilionidae). Journal of Chemical Ecology 22(8): 1411-1428.

Churchill S. 1998. Australian Bats. Sydney: New Holland Publishers.

Cram DJ, Hammond GS. 1964. Organic Chemistry, Second Edition. New York: McGraw-Hill Book Company.

Dechmann DKN, Kamran S. 2005. Studying Communication in Bats. Cognition, Brain, Behavior 9(3): 479-496.

Department of Sustainability and Environment. 2008. About Flying-foxes. Retrieved October 13, 2008, from http://www.dse.vic.gov.au/DSE/nrenpa.nsf/FID/-BAA86C6B029BC723CA256BF2001E4069?OpenDocument#5

Fox S, Luly J, Mitchell C, Maclean J, Westcott DA. 2008. Demographic indications of decline in the spectacled flying fox (*Pteropus conspicillatus*) on the Atherton Tablelands of northern Queensland. Wildlife Research 35: 417-424.

Hagey L, MacDonald E. 2003. Chemical cues identify gender and individuality in Giant pandas (*Ailuropoda melanoleuca*). Journal of Chemical Ecology 29(6): 1479-1488.

Hall L, Richards G. 2000. Flying Foxes: Fruit and Blossom Bats of Australia. Sydney: University of New South Wales Press Ltd.

Lee H, Finckbeiner S, Yu JS, Weimer DF, Eisner T, Attygalle AB. 2007. Characterization of (*E,E*)-farnesol and its fatty acid esters from anal scent glands of nutria (*Myocastor coypus*) by gas chromatography-mass spectrometry and gas chromatography-infrared spectrometry. Journal of Chromatography 1165(1-2): 136-143.

Martin L. 1999. What regulated seasonal breeding in Australian flying-foxes (genus *Pteropus*)? The Australian Bat Society Newsletter 12: 9-11.

Maruani J. 1988. Molecules in Physics, Chemistry and Biology: Molecular Phenomena in Biological Sciences. Chapter: Molecules in Mammalian Communication. New York: Springer.

Nassar JM, Salazarb MV, Quinteroc A, Stonerd KE, Gómez M, Cabrerae A, Jaffe K. 2008. Seasonal sebaceous patch in the nectar-feeding bats *Leptonycteris curasoae* and *L. yerbabuenae* (Phyllostomidae: Glossophaginae): phenological, histological, and preliminary chemical characterization. Zoology 111, 363–376.

Scordato ES, Dubay G, Drea CM. 2007. Chemical Composition of Scent Marks in the Ringtailed Lemur (*Lemur catta*): Glandular Differences, Seasonal Variation, and Individual Signatures. Chemical Senses 32(5): 493-504.

Skoog DA, Leary JL. 1992. Principles of Instrumental Analysis, Fourth Edition. Philadelphia: Saunders College Publishing.

Spencer HJ, Flick BH. 1995. Secretion of marking fluid from the penis of the spectacled flying fox, *Pteropus conspicillatus*. 10th International Bat Research Conference, Boston.

Stander MA, Burger BV, Le RM. 2002. Mammalian exocrine secretions. XVII: chemical characterization of preorbital secretion of male suni, *Neotragus moschatus*. Journal of Chemical Ecology 28(1): 89-101.

Takken W, Dicke M. 2006. Chemical Ecology: from gene to ecosystem. Chapter 1: A multidisciplinary approach. Wageningen, The Netherlands: Wageningen University.

Webb NJ, Tidemann CR. 1995. Hybridization between black (*Pteropus alecto*) and grey-headed (*P. poliocephalus*) flying-foxes (Megachiroptera: Pteropodidae). Australian Mammalogy 18: 19-26.

Welbergen JA. 2004. The social organization of the grey-headed flying fox, *Pteropus poliocephalus*. Cambridge, England: University of Cambridge Press.

Wood WF, Walsh A, Seyjagat J, Weldon PJ. 2005. Volatile Compounds in Shoulder Gland Secretions of Male Flying Foxes Genus *Pteropus* (Pteropodidae, Chiroptera). Zeitschrift Fur Naturforschung C: A journal of biosciences 60(9-10): 779-784.

9.2. Personal Communications

Spencer H. Personal Communication. Director of Cape Tribulation Tropical Research Station. Nov. 3 to Dec. 1, 2008.