UNIVERSITI PUTRA MALAYSIA

MORPHOLOGY OF SALIVARY GLANDS AND ENZYMATIC PROFILES OF WHITE (Aerodramus fuciphagus) AND BLACK (Aerodramus maximus) EDIBLE BIRD'S NEST SWIFTLETS

HELEN ANAK MITIN

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Thesis submitted to the School of Graduate Studies, University Putra Malaysia, in Fulfilment of the requirements for the Degree of Master of Veterinary Science.

July 2013

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I wish to dedicate this thesis to my late father Mr. Mitin Anak Lakid

And my beloved mother Mdm. Mohim Anak Nobek

For their passion and compassion in nurturing

the interest and knowledge by savoring only for the best in their children

The pillar of my strength, Hermisen Anak Ina

For your endless attention, love and devotion in this life

And to my superheroes

Clarisse Ulyssa and Isaac Victor

May this will be your inspiration and motivation for your future endeavours

Abstract of thesis presented to the Senate of University Putra Malaysia in Fulfilment of the requirement for the Master of Veterinary Science

MORPHOLOGY OF SALIVARY GLANDS AND ENZYMATIC PROFILES OF WHITE (*Aerodramus fuciphagus*) AND BLACK (*Aerodramus maximus*) EDIBLE BIRD'S NEST SWIFTLETS

By

Helen anak Mitin

July 2013

Chairman : Intan Shameha Binti Abdul Razak, PhD

Faculty : Veterinary Medicine

Swiftlets are birds within the swift family Apodidae (Genus: Aerodramus) where nine species of *Aerodramus* use their saliva to build nest but only two species produce the edible bird's nest (EBN), the *A. fuciphagus* and *A. maximus*. In Malaysia, *A. fuciphagus* the producer of white EBN are ranched in special houses equipped with bird calling system to attract the bird and with suitable in house temperature, darkness and humidity. The *A. maximus,* the producer of the black EBN is commonly found in the Borneo (Sabah and Sarawak) natural caves. The nest is black because 50 to 60 % of the nest consists of black feathers being glued together by saliva. Since both swiftlets come from the same species, the hypotheses of this study were to show that (1) There is no significance difference in the morphology of salivary glands between *A. fuciphagus* and *A. maximus,* (2) there are different reaction and characterization of enzymes assays between the EBN of *A. fuciphagus* and *A. maximus* and (3) there is no significance difference in the enzymes assays and ultrastructure of the gland between female and male of *A. fuciphagus*. The objectives

of this study were (1) to define the morphology of salivary glands of *A. fuciphagus* and *A. maximus*, (2) to evaluate the presence and intensity of enzymes in the EBN of *A. fuciphagus* and *A. maximus* and (3) to define the ultrastructure of salivary glands in male and female *A. fuciphagus* and determine their intensity of enzymes. The *A. fuciphagus* and *A. maximus* birds were caught in selected areas of Terengganu and Gomantong Caves, Sabah, respectively. A total of 16 swiftlets were used in this study of which eight (8) was *A. fuchipagus* and another eight (8) were *A. maximus.* Birds were sacrificed using 15 mg/kg phenobarbarbitone (Doletal®, Vetoquinol) intramusculary before the salivary glands were removed and processed accordingly for microscopic and enzymatic evaluations. The histochemical staining (Alcian Blue pH 1.0, Alcian Blue pH 2.5, PAS Technique, Alcian Blue- PAS, Alcian Blue-Aldehyde fuschin) and routine Haematoxylin and Eosin staining were used for microscopic evaluations. Comparative enzymatic evaluation was also done on EBN of both species by using a semi-qualititative micro method colour metric test kits. The salivary glands of male and female *A. fuciphagus* were also collected for scanning electron microscope (SEM), transmission electron microscope (TEM) and comparative enzymatic of the salivary glands also was done using the semi-qualititative micro method colour metric test kits. The results showed that the major gland (submandibular gland) was larger and it may vary in size. The glands stained using Alcian blue pH 1.0 and pH 2.5 showed a stronger reaction intensity of all the glangs Alcian blue pH 2.5 indicated that the glands consist of more carboxyl than sulphated mucins. In the lingual gland, the preglottal portion showed stronger reaction than the lingual portion. The glands were stained blue with Alcian Blue-PAS staining indicating the glands were acidic mucin. The Aldehyde Fuschin-Alcian Blue pH 2.5 staining showed the blue discoloration of the glands indicated that the

glands were carboxylated mucin and in PAS staining. Generally all the glands were strongly stained with magenta dicoloration indicating that the presence of glycogen. The salivary glands of both *A. fuciphagus* and *A.maximus* were acidic carboxylated mucin type. Qualitative enzymatic profilling showed that eight (8) types of digestive enzymes detected the in white EBN, while in black EBN there were ten (10) digestive enzymes detected using the ApiZYM®. The Black EBN showed better positive reaction compared to the white EBN. Moderate enzymes intensity (2+ to 4+) was detected for alkaline phosphatase, acid phosphatase and ß-galactosidase. Descriptive ultrastucture evaluation of the salivary glands in both male and female swiftlets showed no morphologically difference. Both sexes of the birds also showed high enzymes intensity (4+ to 5+) compared to a standard color chart and graded on the qualitative 0 to 5+ scale (reactions with reading of >3 (20 nmols) were considered strongly positive; 1 and 2 were considered weakly positive; and 0 was considered as negative/no activity) for alkaline phosphatase, leucinearylamidase, valinearylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, ß-galactosidase and ß-glucuronidase in all oral glands. These studies provides a fundamental knowledge about the anatomical differences of the salivary glands in the white (*A. fuciphagus*) and black (*A. maximus*) and the enzymatic profilling of the salivary gland and EBN which can be used or manipulated to boost the swiftlets industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains Veterinar

Morfologi Kelenjar Air Liur Walit Dan Profil Enzim Sarang Burung Walit Putih (*Aerodramus fuciphagus*) dan Hitam (*Aerodramus maximus*)

Oleh

Helen Anak Mitin

July 2013

Pengerusi : Intan Shameha Binti Abdul Razak, PhD

Fakulti : Perubatan Veterinar

Terdapat sembilan spesis burung walit daripada keluarga *Apodidae* (Genus: *Aerodramus*) yang menggunakan air liur untuk membina sarang tetapi hanya dua spesies yang menghasilkan sarang burung walit (SBW) yang boleh dimakan iaitu *A. fuciphagus* dan *A. maximus*. Di Malaysia, pengeluar putih SBW *A. fuciphagus* diternak di rumah khas yang dilengkapi dengan sistem bunyi untuk panggilan burung, rumah suhu, kegelapan dan peratus kelembapan yang sesuai. Manakala, pengeluar SBW hitam (*A. maximus)* biasanya ditemui di gua semula jadi kepulauam Borneo (Sabah dan Sarawak). Sarang ini berwarna hitam kerana 10 hingga 20% daripada sarang terdiri daripada bulu pelepah hitam yang dilekatkan bersama-sama dengan air liur. Oleh sebab kedua dua burung walit ini adalah dari Genus yang sama, hipotesis bagi kajian ini adalah untuk menunjukkan bahawa (1) tidak ada perbezaan yang signifikan dalam morfologi kelenjar air liur di antara *A. fuciphagus* dan *A. maximus*, (2) terdapat reaksi dan pencirian enzim yang berbeza di antara SBW hitam dan SBW putih (3) tidak ada perbezaan ketara dalam pencirian enzim dan ultrastruktur kelenjar air liur betina dan jantan burung walit *A. fuciphagus*. Oleh yang

demikian, objektif kajian ini adalah (1) untuk menentukan morfologi kelenjar air liur *A. fuciphagus* dan *A. maximus*, (2) untuk menilai kehadiran dan intensiti enzim dalam SBW *A. fuciphagus* dan *A. maximus* dan (3) menentukan ultrastruktur kelenjar air liur dalam jantan dan betina *A. fuciphagus* dan menentukan intensiti reaksi enzim kelenjar air liur tersebut. *A. fuciphagus* dan *A. maximus* telah ditangkap di Terengganu dan Gua Gomantong, Sabah. Sebanyak 16 burung layang-layang telah digunakan di dalam kajian ini di mana lapan (8) adalah *A. fuciphagus* dan lapan (8) adalah *A. maximus*. Burung dikorbankan menggunakan 15 mg / kg phenobarbarbitone (Doletal®, Vetoquinol) secara intramuskular sebelum kelenjar air liur telah dikeluarkan dan diproses sewajarnya untuk penilaian mikroskopik dan enzim. Perwarnaan histokimia (Alcian Blue pH 1.0, Alcian Blue pH 2.5, PAS Teknik, Alcian Blue-PAS, Alcian Blue-aldehid fuschin) dan rutin Haematoxylin dan Eosin telah digunakan untuk penilaian mikroskopik. Penilaian enzim dilakukan pada SBW kedua-dua spesies dengan menggunakan kaedah ujian semi kuantitatif kit mikro warna metrik. Kelenjar air liur jantan dan betina *A. fuciphagus* turut dikumpulkan untuk mikroskop imbasan elektron (SEM), mikroskop elektron transmisi (TEM) dan perbandingan enzim kelenjar air liur juga dilakukan dengan menggunakan kaedah ujian semi kuantitatif kit mikro warna metrik. Hasil kajian menunjukkan bahawa kelenjar utama (kelenjar submandibular) adalah kelenjar yang terbesar dan setiap burung mempunyai saiz yang berbeza. Perwarnaan histokimia Alcian biru pH 1.0 dan pH 2.5 menunjukkan reaksi lebih kuat dengan perwarnaan Alcian biru pH 2.5 pada semua kelenjar yang menunjukkan bahawa kelenjar terdiri daripada kelenjar mucin berkarboksil. Dalam kelenjar lidah, bahagian preglottal menunjukkan reaksi kuat daripada bahagian lingual. Perwarnaan Alcian Blue-PAS pula menunjukkan kelenjar air liur adalah mucin berasid. Perwarnaan Aldehid

Fuschin-Alcian Blue pH 2.5 menunjukkan semua kelenjar berwarna biru yang menunjukkan bahawa kelenjar-kelenjar ini adalah mucin berkarboksi. Perwarnaan dengan PAS pula menunjukkan semua kelenjar diwarnakan dengan warna magenta kerana kehadiran glikogen. Kelenjar air liur kedua-dua *A. fuciphagus* dan *A. maximus* adalah jenis kelenjar mucin karboksi yang berasid. Profil enzim kualitatif menunjukkan bahawa lapan (8) jenis enzim pencernaan dikesan dalam SBW putih, manakala di SBW hitam terdapat sepuluh (10) enzim pencernaan dikesan menggunakan ApiZYM®. The SBW Hitam menunjukkan reaksi yang lebih baik positif berbanding SBW putih. Keputusan intensiti sederhana enzim (2 + 4 +) telah dikesan untuk phosphatase alkali, phosphatase asid dan ß-galactosidase. Penilaian deskriptif ultrastruktur kelenjar air liur burung walit jantan dan betina tidak menunjukkan perbezaan morfologi. Kedua-dua jantina burung juga menunjukkan intensiti enzim yang tinggi dalam semua kelenjar mulut (4 + 5 +) jika perbandingan dengan carta warna standard yang bergred kualitatif 0 ke 5 + skala (tindak balas dengan bacaan> 3 (20 nmols) dianggap amat positif: 1 dan 2 dianggap lemah positif, dan 0 dianggap sebagai negatif / tiada aktiviti) untuk phosphatase alkali, leucinearylamidase, valinearylamidase, asid phosphatase, Naphthol -I-phosphohydrolase, ß-galactosidase dan ß-glucuronidase. Kajian-kajian ini menyediakan pengetahuan asas tentang perbezaan anatomi daripada kelenjar air liur di putih (*A. fuciphagus*) dan hitam (*A. maximus*) dan profilling enzim kelenjar air liur dan EBN yang boleh digunakan atau dimanipulasikan untuk meningkatkan industri burung walit .

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APPROVAL

I certify that a Thesis Examination Committee has met on **4th July 2013** to conduct the final examination of Helen Anak Mitin on her thesis entitled **"Morphology Of Salivary Glands And Enzymatic Profiles Of White (*Aerodramus fuciphagus*) And Black (*Aerodramus maximus*) Edible Bird's Nest Swiftlets"** in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Veterinary Science (Anatomy).

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science (Anatomy). The members of the Supervisory Committee were as follow:

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DECLARATION

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I hereby confirm that:

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- the research conducted and the writing of this thesis was under our supervision;

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LIST OF ABBREVIATIONS

BL	Basal lamina
EBN	Edible bird's nest
ER	Endoplasmic reticulum
GA	Golgi apparatus
M	Mitochondria
Mu	Mucigen
My	Myoepithelial nucleus
MyC	myoepithelial cell
Nuc	Nucleus
N	Demylinated nerve
H&E	Hematoxylin and Eosin
PAS	Periodic Acid Fast Stain
rER	Rough endoplasmic reticulum
Se	Serus granules
sER	Smooth endoplasmic reticulum
SBW	Sarang burung walit
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope

CHAPTER 1

INTRODUCTION

Malaysia is the third biggest exporter of EBN to China after Indonesia and Thailand. Malaysia produces about 137 metric tons of edible bird's nests annually which are equivalent to RM 1 billion according to the Malaysian Bird's Nest Traders Association (Kamarudin *et al*., 2010). According to Agrofood Statistic 2011 by the Ministry of Agricultural and Agro-based industry, there are approximately 4,586 bird houses registered with the Department of Veterinary Services, Malaysia where 4,415 of these houses located in Peninsular Malaysia, 55 houses in Sabah and 116 houses in Sarawak. Since the registration of premises was not compulsory at the moment, the real number of bird houses in Malaysia was estimated more than that.

There are two most common birds that producing the edible bird's nest in Malaysia. The swiftlets of the *Aerodramus sp*. have the ability to secrete the glutinous secretion from the mouth to bind with other materials to build their nest. In Asia, the swiftlets are known to use their saliva as the nest building material during breeding season (Marcone, 2005) and this EBN is used in famous Chinese delicacy bird's nest soup, drinks and cosmetics (Medway 1962b; Koon, 2000; Wong, 2006). The taxonomy of swifts and swiftlets had been disputed for a long time. As cited by Brooke (1970), Mayr (1937) mentioned the reason for this controversy was the high morphological similarity between swiftlet species. Brooke (1970) also cited that there were three main genera in the swift family, which are *Collocalia, Aerodramus* and *Hydrochous*.

There are another nine species of *Aerodramus* that uses their saliva to build up the nest but only two species that produces the edible bird's nest in Malaysia which are *Aerodramus fuciphagus* (*A. fuciphagus*) and *Aerodramus maximus (A. maximus)*.

The *A. fuciphagus*, the producer of white EBN are ranched in non specific designed bird houses equipped with bird's calling system to attract the bird, suitable bird's house temperature, darkness and humidity control system, while *A. maximus* is commonly found in the Borneo island (Sabah and Sarawak states) natural caves. In Sabah, the Gomantong cave (in Sandakan province) is the most productive EBN producer as compared to Madai cave (Tawau Division) which is more for tourist attraction. The nest of the *A. maximus* is blackish in appearance due to the presence of a lot of feathers. *A. maximus* will roost in the nest that was built at the cliffs and clefts in the total darkness of the caves interiors where they can anchor or cement the saliva on the rough surface of the cave's wall. These birds has the ability to navigate in the darken caves through echolocations which are distinctive, penetrating call compose of irregular sharp clicks.

Recently, the swiftlets that colonize new buildings were claimed to come from other established colonies due to such rapid population expansion that likely to affect the genetic variation within the species over a long-term period. The increase of the bird colonies population and possibly genetic variations also due to the increase of number of bird's houses build all over the country. The strong support from the government in the bird's ranching activities as part of the poverty eradication program among the poor and small farmers has stimulated the mushrooming of the bird's house. The bird's nests that are very in sizes and shape which also determine

the quality and grades of the EBN. Each nest takes about 30-45 days to complete in breeding season and in non breeding season it may takes about 60-80 days to complete. The nest is built by both male and female birds at night after spending the day looking for food (Nughoro and Wendrato, 1996). According to Lim and Cranbrook (2002), there was no intraspecific seasonal variation between nests constructed at different month of the year in white EBN, but there were significant differences in chemical constituents of carbohydrate in *A. maximus* and *A. fuciphagus*. Their field observation and anatomical examinations have shown that the saliva was produced from the mouth of both sexes during nest building.

The saliva is actively produced by a pair of salivary glands located at the floor of the mouth with the ducts open on the mucosa surface of the submandibular region. However, the type of enzymes in the salivary glands of EBN swiftlet's and their presence in the dried EBN are still unknown. As cited by McLelland 1975, the morphology of the salivary glands in birds has been studied for decades (Greschik, 1913; Bock, 1961) and the results showed that these glands are functional and efficient in birds which feed mainly on small insects or seed, less developed in those eating soft diet, and absent in some birds such as pelican (King and McLelland, 1984; Blanks, 1993). Grossly the salivary glands of black-nest swiftlet (*Aerodramus maximus*) were found relatively bigger during breeding season compared to the non breeding season (Lim and Cranbrook, 2002). In addition, Marshall and Folley (1956) and Medway (1962a) also reported that these glands fluctuated in size and activity, becoming enlarged during periods of nest building and diminished at other times, but this statement is not conclusive as the enlarged glands is not diminished but may

reduced in size. However, no histochemical evaluation of the salivary glands has been reported up to date on the salivary glands of *A. maximus* and *A. fuciphagus.*

There were broad and growing interests in knowing more about the components and the nutritional and medicinal values of the EBN. The present study of the EBN was expected to provide references for farmers, consumers and future researchers. The EBN is believed to have significant nutritional and medicinal values such as the potential for mitogenic response, epidermal growth factor (EGF)-like activity, anti-influenza virus, hemagglutination-inhibitory activity, lectin binding activity, improvement of bone strength and dermal thickness, and hormone content. The earlier study reported that the main component of the EBN includes 32.3% water-soluble proteins, 38.7% carbohydrates, 16.9% hexose, 12.5% hexosamine, 0.7% fructose, 8.6% sialic acid and 20% inorganic ash (Kathan and Weeks, 1969). The 8.6% sialic acid activity has been used to study the sialidase activity of influenza virus (Howe *et al*, 1961b), myxovirus heamagglutination inhibitor and viral neuraminidase (Howe *et al*, 1961a). It was found that EBN extract could strongly inhibit infection with influenza viruses in a host range-independent manner (Howe *et al*, 1961b). A study by Wieruszeski *et al*. (1987) showed that the nest cement-like structure or the mucoid glycoprotein was mainly made of sialic acid rich in O-glycosylproteins. They were incorporated in various kinds of food products, including drink and food additives and also used as a cosmetic ingredient. There is dearth information related to the evaluation of the EBN swiftlet's salivary glands, and since the salivary glands of this birds has a special function which are not just related to their diet but also in the production of the precious EBN, this study was conducted to understand the function of the most important organ in black (*A.*

fuciphagus) and white (*A. maximus*) EBN swiftlets which are the salivary glands. Fucui and Daicheng (2012) had sketched the importance and bioactivities of the edible bird's nest but mainly focus on the EBN and the product of EBN. The unavailablity of the information in the literature on the morphology of the salivary glands of these birds had led to the in-depth study of the salivary glands. The possibility of the digestive enzymes present in the dried EBN and the type of enzymes in the salivary glands are still unknown.

The importance of this study was to have a good fundamental understanding of the anatomical and enzymatic profilling of the salivary glands of the EBN swiftlets. The preliminary qualitative study of the ezymatic profiling in EBN itself also helps in recognising the existance of the digestive enzymes which may be of an importance in the near future to evaluate the physiology and the efficiency of the digestive system in swiftlets. This information and knowledge will be the fundamental reference that can be used to get a sustainable bird population and edible bird's nest industry in the country. In this study, the hypotheses were

1. There is no significance difference in the morphology of salivary glands between *A. fuciphagus* and *A. maximus.*
2. There are different reactions and characterization of enzymes assays between the Edible bird's nest of *A. fuciphagus* and *A. maximus.*
3. There is no significance difference in the enzymes assays and ultrastructure of the gland between female and male of *A. fuciphagus.*

The objectives of this study were:

1. to study the morphology of salivary glands of *A. fuciphagus* and *A. maximus*
2. to determine the enzymatic profile in the edible bird's nest (EBN) of *A. fuciphagus* and *A. maximus*
3. to study the ultrastructure of salivary glands in male and female *A. fuciphagus* and to determine their enzymatic profile.

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