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Chemical Defence in the
Red-billed Woodhoopoe,
Phoeniculus purpureus.

Janette Law - Brown

**Submitted in fulfilment of the degree of Master of
Science by dissertation, Percy FitzPatrick Institute,
Department of Zoology, University of Cape Town**

April 2001

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Declaration:

This thesis reports the results of original research I conducted under the auspices of the Percy FitzPatrick Institute of African Ornithology, Department of Zoology, University of Cape Town, between 1999 and 2001. All assistance that I received has been fully acknowledged. This work has not been submitted for a degree at any other university.

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Janette Law-Brown

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Abstract:

Red-billed Woodhoopoes, *Phoeniculus purpureus*, produce a pungent smelling secretion from their uropygial gland. Previous researchers have noted this odour and there has been much speculation on its function. This encouraged me to undertake this study to determine the origin of the odour and the role that the secretion plays. The chemical analysis of this secretion shows that it consists of 17 compounds including acids, aldehydes, lactones and other miscellaneous compounds. Cultures of the secretion showed the presence of a symbiotic bacterium resident within the gland. Antibiotic treatment of the gland suggested that this bacterium was instrumental in the synthesis of the secretion of *P. purpureus*. This bacterium has not previously been identified and has been proposed as '*Enterococcus phoeniculicola*' (GenBank accession number: AY028437). The secretion was synthesized and the responses of a range of 13 pathogenic and one parasitic bacteria were tested against it and its individual constituent compounds. The synthetic secretion and seven of its constituent compounds inhibited the growth of these bacteria. However, exposing feather mites resident upon the birds to the synthetic secretion did not illicit any significant response from these mites. Wanless (1998) showed that the synthetic secretion causes an averse reaction in cats. In this study I was able to identify indole, phenol and propionaldehyde as being responsible for eliciting this reaction in cats. A similar averse reaction is shown by Rock Monitor Lizards, *Varanus albigularis*, upon encountering mice tainted with the synthetic secretion. This is the first demonstration of a symbiotic relationship between birds and bacteria in which the modification of the birds' uropygial oils affords the birds some protection against pathogens and predators.

Acknowledgements:

To my supervisor, Professor Morné Du Plessis who saw the possibilities of this study and allowed me free reign with my ideas. Similarly, to Dr Paul Meyers of the Department of Molecular and Cell Biology, University of Cape Town, for allowing to make use of his laboratory and his invaluable help with the microbiological side of this project.

To Professor Ben Burger of the Department of Chemistry, University of Stellenbosch, for his analysis of the Red-billed Woodhoopoe secretion, for the synthesis of the artificial secretion and providing me with constituent compounds. To Ross Wanless for allowing me to use his data on cat reactions to the synthetic secretion. To Dr Reena Cotton of the Blue Cross Animal Hospital, Newlands, for supervising the antibiotic treatment of the woodhoopoes. To Dr Andrew Jenkins, Giselle Murrison and Patrick Brackley for their help in collecting samples from the birds. To John Clausen of the Microbiology Department of the Red Cross Children's Hospital for his help in determining lactic acid production by '*E. phoeniculicola*'. To Di James of the Department of Molecular and Cell Biology, University of Cape Town, for doing the 16S rDNA sequencing of '*E. phoeniculicola*'. To Neil Madgwick of Allerton Provincial Veterinary Laboratory in Pietermaritzburg, KwaZulu – Natal for providing me with bacterial cultures for use in the inhibition zone experiments. To the laboratory staff ARC-Plant Protection Research Institute, Pretoria, for identifying the feather mites found on the woodhoopoes. To The Society for the Prevention of Cruelty to Animals, Grassy Park, The Worcester Snake Park and Die Vonds, Paarl, for allowing me access to their animals for experimental purposes.

For their help during the writing up process I would like to thank the following people: Professor Morné Du Plessis (for carefully going through all my chapters repeatedly), to Dr Paul Meyers (for helping me with chapters 3 and 4), Professor Phil Hockey and Raurie Bowie (for their comments on my conclusion), Antje Leseberg (for translating articles for me), and Dr Barry Clark and Dr Justin O Riain (for their help in statistical matters).

I also very grateful to the staff at the FitzPatrick Institute for their help in making this project run as smoothly as possible. To my family and friends – for supporting me throughout this project, especially when things didn't seem to be going as they should be!

Finally, to the National Research Foundation, without whose financial contribution this study would not have been possible.

I. Introduction:

The use of chemical compounds by animals for defence and communication has been noted in many organisms. Chemical defence occurs "...when an individual contains or uses behaviourally one or more chemical substances that deter predators and / or parasites." (Dumbacher and Pruett-Jones 1996). Many species of invertebrates are known to produce toxins and acids as a form of defence (Eisner *et al* 1965, Lawrence 1981, William *et al* 1997). Vertebrates such as poison-dart frogs (*Phyllobates aurotaenia*) (Albuquerque *et al* 1971) and other amphibians also produce toxins and irritants to dissuade predators. In mammals chemical compounds are mostly used for communication (Kappeler 1998, Burger *et al* 1999). Very few mammals, such as the skunk and stripe-necked mongoose, produce (Burton 1976) chemicals for defence.

Many species of birds are known to be malodorous, toxic or unpalatable (Cott 1945, Cott 1946, Cott 1953, Dumbacher and Pruett-Jones 1996). One such species is the Red-billed Woodhoopoe, *Phoeniculus purpureus*, which, like other species of woodhoopoe (Steyn 1999) carries a very distinctive odour attributable to the secretion produced in the uropygial gland of these birds. The question arises of how this odour is produced. Three likely methods for the origin of chemicals in animals are recognised: *de novo* synthesis, sequestration of chemicals directly from the diet, and the production of chemicals from bacterial gut fermentation. Birds are known to sequester toxic chemicals from their insect prey and from plants (Cott 1946, Dumbacher and Pruett-Jones 1996). The production of repellent chemicals through bacterial gut fermentation is well documented in humans (al-Waiz *et al* 1992, Rehman 1999) and has been documented in birds (Dumbacher and Pruett-Jones 1996). The only birds thought to

produce toxins through *de novo* synthesis are of the genus *Pitohui* (Dumbacher *et al* 1992).

Once the question of the origin of the odour of the woodhoopoes has been addressed another question arises: What role does it fulfil? As was stated earlier, mammals use chemicals for communication, however most birds are not known to have a well developed sense of smell (Burton 1976, Mason and Clark 1998, Rangen *et al* 2000). If the secretion is not used for communication amongst the birds, the only feasible role it can fulfil is that of protecting the birds. Birds play host to a whole suite of symbionts, be they pathogens, parasites, mutuals or commensals. Many species of bacteria have been identified living within and on birds, as well as from their nest sites (Cooper 1990, Cooper 1993, Hubálek *et al* 1995, Singleton and Given Harper 1998, Burt 1999). Many of these bacterial species make up part of the normal flora of the birds, yet some may be opportunistic or obligate pathogens.

In addition to carrying a burden of potentially lethal micro-organisms, birds also play host to a range of arthropod symbionts. In birds with long bills, deformed bills or feet, or those that are compromised immunologically, such arthropod populations could swell to numbers that might become dangerous to the health of the birds (Clayton 1991). Increased arthropod ectoparasite numbers present a threat not only to adult birds (Clayton 1990, Clayton 1991b), but also nestlings (Dobson and Hudson 1986, Threlfall 1986).

Birds face threats not only of a microscopic nature, but also of a macroscopic nature. Mammals, reptiles and raptors routinely depredate birds (Cott 1953, Pitman 1962, Pitman 1962b, van Aarde 1980, Dyer 1996). It is not likely that a malodorous secretion

would have much effect upon raptors, but animals that smell their prey and often raid nest cavities would likely be deterred by malodour that might indicate an unpalatable prey species.

Birds have also been observed rubbing arthropods through their feathers. This is usually done with ants that produce acids and other volatile compounds (Judson and Bennett 1992, Clayton and Vernon 1993, Clayton and Wolfe 1993). Birds have also been observed selecting specific plant species that produce volatile chemicals for incorporation in their nest material (Clark and Mason 1985, Mason and Clark 1986, Clark and Mason 1988). These plant species inhibit certain stages in larval ectoparasite growth, as well as that of bacterial growth. Monteiro's Hornbills (*Tockus monteiri*), and other African hornbill species, collect millipedes which are then crushed and placed in the birds' nest material and in the material used to seal the nest cavity. Millipedes secrete benzoquinones and hydrogen cyanide from repugnatorial glands present along the lateral sides of the body (Krebs 1964, Eisner *et al* 1965, Lawrence 1981, Attygalle *et al* 1993, Williams *et al* 1997). This secretion apparently protects the millipedes against predators, pathogens and parasites (van der Walt and McClain 1990, Williams *et al* 1997). This poses the question: are the hornbills attempting to 'fumigate' their nest cavities in an attempt to improve their breeding performance?

In this study I attempt to determine how the characteristic secretion of the Red-billed Woodhoopoe is produced (chapter 2). Further, I test whether the secretion fulfils an antibiotic role, whether it helps reduce the arthropod load of the birds (chapter 4), or whether it protects the birds from mammalian and reptilian predators (chapter 5). In addition, I examine the behaviour of the Monteiro's Hornbills in placing crushed

millipedes in their nest material (appendix A), so as to determine if this is done to reduce the bacterial and fungal populations of the nest boxes prior to the birds breeding.

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II. The Origins of the Characteristic Odour of Red-billed (Green) Woodhoopoes, *Phoeniculus purpureus*.

Summary:

The uropygial secretion of the Red-billed Woodhoopoe, *Phoeniculus purpureus*, consists of 17 compounds, including acids, aldehydes, lactones and other miscellaneous compounds. The secretion of wild birds does not differ significantly from the secretion of birds held in captivity and fed on an artificial diet. The uropygial gland of *P. purpureus* contains a symbiotic bacterium. When the gland of the birds is flushed with antibiotic for a period of three days the bacterium is eliminated and the number of compounds within the secretion drops to seven. Only two of the compounds in the sterile secretion are the same as those in the original secretion, yet their concentrations are vastly different. Apparently the uropygial secretion of the Red-billed Woodhoopoe is not sequestered from the birds' food, nor is it synthesized *de novo* by the birds. Instead, it is a product of the metabolism of the bacterium resident in the birds' uropygial gland upon the uropygial oil produced by the birds. This is the first clear demonstration of this type of symbiotic relationship.

Introduction:

Almost all birds have an uropygial gland from which they produce a secretion used in preening; the Struthioniformes is the only order of birds known not to have even a vestigial uropygial gland (Jacob 1982). The preen glands of birds are considered analogous to the sebaceous glands found in mammals (Asnani and Ramachandran 1993) and secrete a variety of chemical compounds. The primary chemicals secreted by the gland appear to be monoester and diester waxes, alcohols and squalene (Jacob 1982, Levy and Strain 1982, Jacob and Hoerschelmann 1985, Livezey *et al* 1986). The chemical composition of the secretion generally reflects the environment in which the birds live (Livezey *et al* 1986).

The primary function of the uropygial gland and its secretion seems to be for the maintenance and, in the case of marine birds, waterproofing of feathers (Jacob 1982, Levy and Strain 1982, Jacob and Hoerschelmann 1985, Livezy *et al* 1986). Another possible function of the uropygial gland secretion could be to deter predators, as in the Eurasian Hoopoe that secretes a noxious substance from its uropygial gland when threatened (Elder 1954). Birds such as the *Pitohui* spp. produce toxic chemicals within their flesh and feathers and the birds' bodies have a musky, unpleasant odour (Dumbacher *et al* 1992). Red-billed Woodhoopoes and Scimitar-billed woodhoopoes produce a malodorous secretion from their uropygial secretion (Ligon and Ligon 1978, Steyn 1999). Many mammals have scent glands located in various areas of their bodies that are used in communication. Mammalian scent glands are used in territorial marking, as an indication of group status, sexual status or as a predator deterrent (Burton 1976, Kappeler 1998, Burger *et al* 1999).

Birds are known to sequester repellent chemicals from their diet. The Great Spotted Woodpecker, Eurasian Green Woodpecker and the Red-tailed Ant-Thrush sequester chemicals from their insect diet (Cott 1946, Ziegler 1971) while birds such as the Kalij Pheasant and Olive Pigeon sequester chemicals from their plant foods (Dumbacher and Pruett-Jones 1996). Malodorous chemicals may also be sequestered from bacterial gut fermentation. In humans compounds such as trimethylamine are products of gut fermentation and are then released from the body in sweat and urine (al-Waiz *et al* 1992, Rehman 1999). The Hoatzin derives its odour partly from fermentation by the bacteria within its gut. In New Guinea the genus *Pitohui* has been documented as a toxic bird species (Dumbacher *et al* 1992). This birds apparently synthesizes the toxin found in its feathers, skin and muscle tissue *de novo* as the only other source of

homobatrachotoxin is from the poison-dart frog (*Phyllobates*) found in parts of Central and South America (Albuquerque *et al* 1971).

I aim in this study to determine whether Red-billed Woodhoopoes, *Phoeniculus purpureus*,

- sequester compounds from their diet
- sequester secondary metabolites from their gut flora
- employ *de novo* synthesis

in the production of their characteristic uropygial secretion.

Methods:

Red-billed Woodhoopoes, *Phoeniculus purpureus*, commonly occur in the mesic woodlands in the eastern parts of Africa. Woodhoopoes are obligate communal roosters, that roost and breed cooperatively in cavities (Ligon and Ligon 1978a, Du Plessis and Williams 1994). Groups size ranges from two to 12 members and the group is comprised of a single breeding pair and a number of non-breeding helpers (Ligon and Ligon 1978b, Du Plessis 1993). Woodhoopoes are insectivorous, using their long curved bills to prise insects out from under the bark of trees (Steyn 1999). They will also take very small quantities of vegetable matter, such as *Strelitzia* seeds.

I collected samples of the uropygial secretion of *P. purpureus* from wild birds caught in the Morgan Bay area of the Eastern Cape, South Africa (32° 43'S, 28° 47'E) between 21/04/1999 and 26/04/1999. I collected these samples using sterile capillary tubes,

which I then stored in sterile eppendorf tubes sealed with ParafilmTM. A group of six birds, three females and three males, were captured and transported to the University of Cape Town, on 25/07/1999, where they were housed in outdoor aviaries. I took further uropygial secretion samples after the birds had been held in captivity for one month (30/08/1999). The birds were fed a diet of minced ox heart, mealworms and AviPlus[®] for soft bills, and maintained weight and condition throughout their period of captivity (figure 2.1). The number of compounds found within the samples were compared using the T-test for independent samples (Zar 1996).

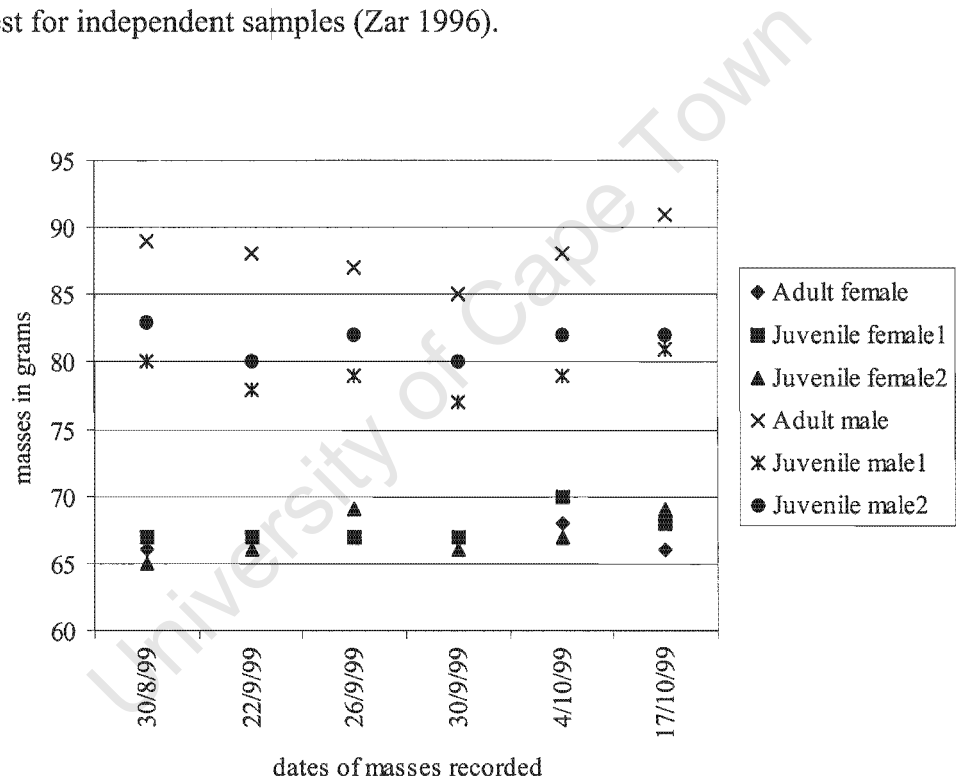


Figure 2.1: The changes in body mass shown by the birds during their period of captivity and the respective treatments.

I took uropygial samples from the birds to determine whether or not bacteria were present in the gland. I separated the birds into two groups, one containing one male and

two females (control) and the other containing one female and two males (treatment). Once I had separated the birds into two groups they were placed in adjacent aviaries, which are separated by fine mesh. Uropygial samples were taken to establish that the chemical composition of the uropygial secretion was the same in all the birds in both groups. Treatment was treated with 0.2ml of BaytrilTM (Enrofloxacin, manufactured by Bayer), a broad spectrum antibiotic that was injected directly into the uropygial gland of the birds. This was done to prevent the antibiotic from affecting any of the birds' other normal flora. Control was treated in the same manner, using sterile saline instead of antibiotic. This process was repeated for three consecutive days to ensure that the bacteria in the gland had been exterminated. A qualified veterinarian was present during all antibiotic administrations. During the period of the administration of the antibiotic I took daily uropygial samples for bacterial analysis. I took further uropygial samples two days after the final administration of the antibiotic for chemical analysis. Samples for bacterial analysis were taken every three days for a further four weeks after the final administration of the antibiotic. A final sample was taken for chemical analysis 33 days after the final administration of the antibiotic. I placed the bacterial samples in 1ml of sterile water and diluted this solution up to 10^{-3} ; 100 μ l of each dilution was spread plated in duplicate onto Biolab[®] nutrient agar plates and incubated overnight at 37°C. I recorded the number, shape and colour of the colony-forming units (CFU's) of these cultures and drew a comparison between the cultures of the secretion from the birds treated with antibiotic and those treated with saline. The chemical samples taken during the antibiotic treatment were compared using the Lord's Range test (Langley 1968), due to the small sample size.

I sent the uropygial samples extracted for chemical analysis to the University of Stellenbosch. The samples were analysed using the methods of Burger *et al* (1999), with exception of the sample preparation procedure. The sampling time was seven hours at room temperature before the samples, after cryo-focussing, were subjected to GC-MS analysis. Extraction of the secretion was prepared by exposing the samples to as little dichloromethane as possible and allowing them to stand for two hours, after which the samples were centrifuged in sintered glass filter-vials at 2000 – 3000 rpm for approximately five minutes. The dichloromethane extract was removed from beneath the supernatant water and mucous layer using a 100ml syringe and transferred to clean Reacti-Vials. Most of these extracts could be used without further concentration. However, where this was not the case, the solvent was removed in a slow stream of purified (activated charcoal) nitrogen.

Results:

The chemical composition of the samples taken in the field was compared with those taken in the aviary one month after the capture of the birds. There was no difference between the number of chemical constituents within the secretion samples taken in the field and those samples taken after the birds had been held in captivity for one month ($p = 0.544$, $t_{crit} = -0.627$).

A significant difference in the number of compounds was found between the secretions of groups A and B on day 4 ($L = 2.3$, $p < 0.05$) and on day 36 ($L = 3$, $p < 0.05$). No difference in the number of chemical constituents was found on 9/11/1999, before the administration of the antibiotic ($L =$). Table 2.1 shows the chemical composition of the *P. purpureus* secretion before the antibiotic treatment and after the antibiotic

Table 2.1: The chemical composition, and relative concentrations, of the uropygial secretion of the captive Red-billed Woodhoopoes, *Phoeniculus purpureus*, before treatment with the antibiotic Baytril (pre-treatment) and the chemical composition of the captive woodhoopoe secretion after the antibiotic treatment (post-treatment).

<u>Compounds:</u>	<u>Concentration (%):</u>	
	<u>Pre - Treatment:</u>	<u>Post - Treatment:</u>
Propionaldehyde	0.5	0
Butyraldehyde	0.2	0
Isovaleraldehyde	22.6	3.4
Heptaldehyde	2.5	0
Benzaldehyde	8.4	0
Propionic acid	0.5	0
Isobutyric acid	22.6	0
Valeric acid	0.9	0
Hexanoic acid	0.2	0
Benzoic acid	28.1	0
γ -Butyrolactone	7.1	0
σ -Valerolactam	0.9	0
Hexanal	2.9	0
Dimethyldisulfide	0.4	12.0
Phenol	0.7	0
Indole	4.5	0
Trimethylamine	2.1	0
1 - Butanol	0	5.7
Methoxybenzene	0	2.8
Limonene	0	2.9
Pyrazine derivative	0	12.5
Cholesterol	0	62.7

treatment. The relative concentrations of the constituent compounds are also indicated. Figure 2.2 shows the trends in the number of chemical constituents found within the secretion of the two groups of birds prior to the treatment (day1), two days after the final treatment (day 4) and one month after the final treatment (day 36). At the time of taking the final sample for chemical analysis the number of chemical constituents in the secretion of treatment was still less than that of control. The secretion of treatment tended to be paler, more fluid and less sharp in odour than that of the birds of control.

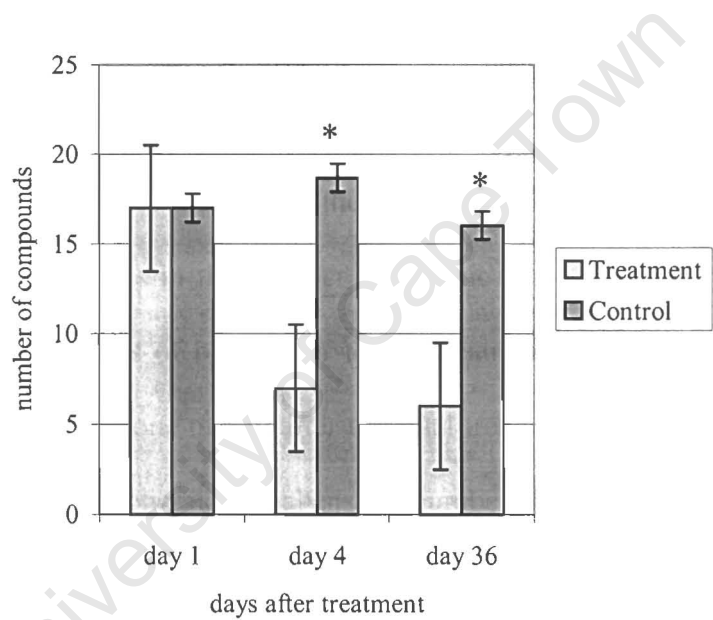


Figure 2.2: Changes in the number of chemical compounds found within the secretions of those *P. purpureus* treated with sterile saline (control) and those treated with Baytril (treatment).

* denotes a significant difference (Lord’s Range Test: $L > 0.64$, $p < 0.05$)

During the time of the antibiotic treatment cultured samples of the secretion showed that the micro-flora resident in the gland had been destroyed. Secretion samples were

cultured every three days for 27 days after the antibiotic treatment. After 24 days microbial growth was found in all of the secretion samples of both groups A and B.

Discussion:

Under natural conditions *P. purpureus* feeds predominantly on insects that are retrieved from below the bark of trees. The captive birds were fed on meat and a commercially available dietary supplement for insectivorous birds. Birds are known to sequester chemical compounds from their diet, be it insect or plant (Cott 1946, Dumbacher and Pruett-Jones 1996). The chemical composition of the natural and artificial diets of *P. purpureus* have not been compared. Thus the sequestration of compounds from the diet cannot be discarded. However, evidence that argue against this in the case of *P. purpureus*, is that there was no difference between the number of compounds found in the secretion of birds that changed from a natural diet to an artificial diet in captivity. There was also no noticeable difference in the smell, texture, or colour of the secretion.

In all cases where chemical compounds have been reported to have been synthesized by bacterial symbionts, these symbionts have been gut flora (Cott 1946, Dumbacher and Pruett-Jones 1996). Bacteria are known to produce a number of aromatic compounds through the metabolism of their substrates (Allison 1978, Camargo *et al* 1982, Padilha *et al* 1995, Cabral *et al* 1997, Krings and Berger 1998, Arp 1999, Khan *et al* 1999, Taniguchi *et al* 1998, Rehman 1999, Birtkett *et al* 2000, Cass *et al* 2000, Higaki *et al* 2000, Rose *et al* 2000). Of the original 17 compounds found in the uropygial secretion of the Red-billed Woodhoopoe only two, dimethyl disulfide and isovaleraldehyde, were still present in the secretion after the birds had been treated with antibiotics. Topical application of the antibiotic was necessary to distinguish between compounds that were

formed by the bacteria in the gland and any compounds that may be absorbed from bacterial action in the gut. Although isovaleraldehyde and dimethyl disulfide are present in the secretion when there is no bacterial symbiont present, their relative concentrations are far different to what they would be naturally. The concentration of dimethyl disulfide is far higher in the sterile secretion than it would be under normal circumstances. This suggests that these compounds are being sequestered from the birds' diet into the uropygial secretion and then partially metabolised by the symbiotic bacterium resident in the uropygial gland. The concentration of isovaleraldehyde is much lower in the sterile secretion suggesting that the bird is producing, or sequestering, a small amount of isovaleraldehyde, but that the bulk is produced by the metabolism of the resident bacterium. When the secretion is not being modified by the microbial symbiont, cholesterol is the primary component of the secretion. This is in accordance with previous research that shows that uropygial oils seem to be mostly comprised of fats and waxes (Jacob 1982, Levy and Strain 1982, Jacob and Hoerschelmann 1985, Livezy *et al* 1986). By comparing the concentrations of the chemicals produced by the birds in the secretion with the concentration and number of chemicals found within the secretion when the microbial symbiont is present, it appears that most of the chemicals found within the natural secretion are metabolites of cholesterol.

Birds of the genus *Pitohui* are presently the only birds thought to synthesize toxins *de novo* (Dumbacher *et al* 1992). From the analyses of the secretion produced by the woodhoopoes, the only compound that might be produced *de novo* by the birds is isovaleraldehyde. Dimethyl disulfide may be sequestered from the birds' diet, but the other 15 compounds that comprise the secretion, with DDS and isovaleraldehyde, are

being produced by the bacterium which is resident within the uropygial gland of the woodhoopoes.

My findings lead me to believe that the production of this secretion is indeed dependant upon a symbiotic relationship between birds and bacteria. It is, however a symbiotic relationship which has not previously been described.

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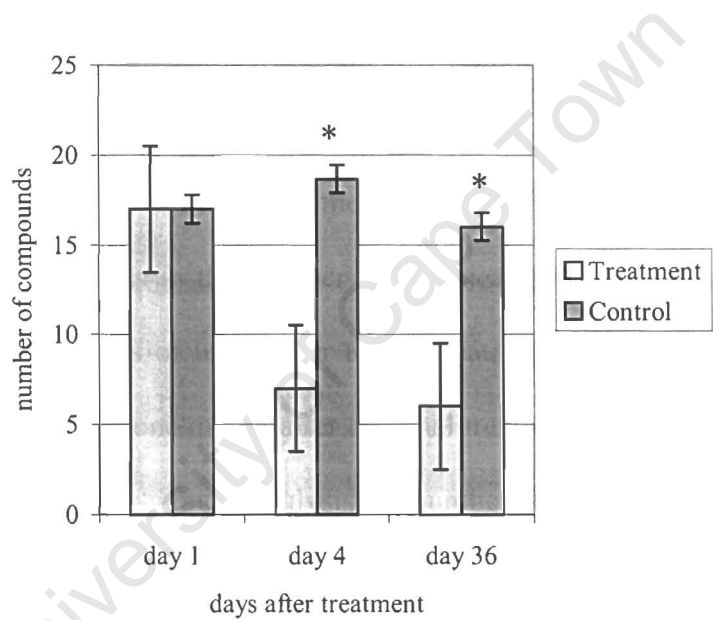


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III. Description of the symbiotic bacterium,

'Enterococcus phoeniculicola' sp. nov., resident

within the uropygial gland of the Red-billed

Woodhoopoe, *Phoeniculus purpureus*.

Summary:

A facultatively anaerobic, Gram positive, non-sporing, non-motile, catalase-negative bacterium was isolated from the uropygial gland of wild Red-billed Woodhoopoes, (*Phoeniculus purpureus*) and designated strain JLB-1. Physiological and biochemical testing indicated that this species is a homofermentative, lactic acid producing member of the genus *Enterococcus*, following the current definition of this group. The 16S rDNA sequence was compared with other sequences using the BLAST facility of GenBank. The closest relatives of the bacterium are *E. avium*, *E. faecium* and *E. asini* (96%). Strain JLB-1 may be differentiated from its closest relatives by the fact that it does not produce acid from lactose, mannitol, melezitose or sorbitol. It also does not hydrolyse arginine or hippurate and cannot grow in 6.5% NaCl nor 40% bile. The name *Enterococcus phoeniculicola* sp. nov. is proposed for this species. The type strain is JLB-1. The 16S rDNA sequence has been submitted to GenBank (accession number AY028437).

Introduction:

Prior to the genus *Enterococcus* being proposed by Schleifer and Klipper-Bälz (1984), this group of organisms was a subdivision of the streptococci (Hardie 1986). Currently the genus *Enterococcus* is divided into four groups, comprising 20 species, based upon 16S rDNA sequence homology. Phylogenetically the enterococci belong to the clostridial sub-division of the Gram positive bacteria and cluster with *Vagococcus*,

Tetragenococcus and *Carnobacterium* (Vancanneyt *et al* 2001). Physiologically the enterococci are related to the streptococci by their ability to grow at 10°C and 45°C, at pH 9.6, in 6.5% NaCl and 40% bile (Schleifer and Klipper-Bälz 1984). It has been discovered since the establishment of the genus that not all enterococci comply with this definition (Devriese *et al* 1983, Devriese *et al* 1990). The analysis of the 16S rDNA gene sequence of enterococci has also played a large part in the differentiation of streptococci from enterococci (Devriese *et al* 1990).

Streptococci are often found as symbionts of humans and animals, be they parasitic (Kawamura *et al* 1998, Devriese *et al* 1999, Rurangirwa *et al* 2000, Schlegel *et al* 2000, Zhu *et al* 2000) or apparently commensal (Devriese *et al* 1997). Streptococci have also been identified which do not live on or in human or animal bodies (Sly *et al* 1997, Flint *et al* 1999). However, whether streptococci are isolated from humans, animals, or other sources, they are haemolytic and their growth is encouraged by the presence of blood in the culture medium (Hardie 1986). Enterococci are also known to be opportunistically pathogenic (de Vaux *et al* 1998, Hsueh *et al* 2000).

Burger *et al* (1999) found *Bacillus brevis* and *Planococcus citreus* in the interdigital secretion of the bontebok (*Damaliscus dorcas dorcas*) and the blesbok (*D. d. phillipsi*). As yet there is no evidence as to whether these bacteria modify the secretions of these antelope or not. Many species of bacteria and fungi have been isolated from birds, their nests and their alimentary canals (Hubálek 1976, Cooper *et al* 1986, Cooper 1990, Cornelissen *et al* 1991, Cooper 1993, Hubálek *et al* 1995, Singleton and Given Harper 1998, Burt and Ichida 1999). In most of these cases the bacteria and fungi isolated have been associated with disease and not with any function that could possibly be

beneficial to the bird (see chapters 3 and 4). Recently a Gram positive, non-motile, non-sporing coccus was isolated from the uropygial secretion of the Red-billed Woodhoopoe, *Phoeniculus purpureus*. The bacterium was found to modify the uropygial secretion produced by the birds through its own metabolism (see chapter 1).

Methods:

Physiological and biochemical tests:

I collected samples from the uropygial gland of the Red-billed Woodhoopoe, *Phoeniculus purpureus*, using a 100µl autopipette. Tips containing the samples were placed in sterile MacCartney bottles. I flushed the samples into MacCartney bottles containing sterile nutrient broth (Biolab Diagnostics[®]) and incubated these overnight with agitation at 37°C. From these samples I performed the Gram stain, the endospore stain and inoculated nutrient agar (Biolab Diagnostics[®]) plates, on which the bacterium was maintained throughout the identification process. I tested the bacterium's ability to grow on nutrient agar containing 40% bile salts, 0.5%, 3% and 6% NaCl. I tested the bacterium's ability to grow at pH 4.5, pH 6, pH 8 and pH 9 by preparing nutrient agar and adjusting it to these pH's. The growth of the bacterium was also tested in nutrient broth containing 10 % ethanol. The bacterium was tested for hippurate hydrolysis using the method described by Gordon and Barnett (1974), lipid hydrolysis using Sierra's medium, aesculin hydrolysis as per the method described in Bergey's Manual of Determinative Bacteriology (1982) and arginine hydrolysis (Palleroni 1986). Acid and gas production from sugars was performed in peptone water containing 0.5% of the test

sugar in capped test tubes containing a Durham tube. The tubes were incubated at 37°C for 24 hours without agitation. The sugars that were tested were: L(+)arabinose, D-cellobiose, β -cyclodextrine, dulcitol, D(-)fructose, D(+)galactose, D(+)glucose, *meso*-inositol, inulin, α -lactose, maltose, D-mannitol, D(+)melezitose, melibiose, raffinose, L(+)rhamnose, D(-)ribose, salicin, D-sorbitol, L(-)sorbose, sucrose, trehalose and D(+)xylose. I also tested glycerol and sodium succinate in this manner for acid and gas production. I tested the bacterium for acetoin production (Voges – Proskauer test), nitrate reduction, indole production, citrate utilisation, gelatine liquefaction, casein hydrolysis and acid production (Methyl Red – Voges Proskauer test). I also tested the reaction with litmus milk.

To test whether strain JLB-1 is a lactic acid bacterium, the bacterium was cultured for 24 hours in nutrient broth at 37°C with agitation. The bacteria were harvested by centrifugation, the supernatant was filter sterilised and was then tested for lactic acid production using a Chrompak model 437A gas chromatograph. Samples were prepared for analysis for volatile fatty acids and non-volatile acids (see appendix for methods).

I tested strain JLB-1 for haemolysis using blood agar. I also tested the bacterium's ability to grow on minimal media using M9 medium, M9 medium supplemented with casitone (Difco laboratories) and M9 medium supplemented with yeast extract. I tested for DNase activity using DNase agar (Oxoid[®]). The recipes for the various media used may be found in the appendix. The blood agar plates were obtained from Media Services, Medical Microbiology, University of Cape Town.

PCR analysis:

I amplified the 16S rDNA gene of strain JLB-1 using universal primers for the amplification of bacterial 16S rDNA genes (F₁: 5' AGAGTTTGATCITGGCTCAG 3'; R₅: 5'ACGGITACCTTGTTACGACTT 3'). The amplified DNA was then sequenced using the ALFexpress DNA Automated sequencer and the Amersham Pharmacia Biotech Cy5TM Thermo Sequenase Dye Terminator Kit. The chain termination technique of Sanger *et al* (1977), which employs dideoxy cycle sequencing reactions using Thermo SequenaseTM, was used. All reactions were performed according to the manufacturer's instructions. The DNA was amplified using the Biometra[®] Personal Cycler. Electrophoresis was done using Reprogel Long Read (7%) and was run according to the manufacturer's operating procedure for 13 hours at 55°C, using a standard gel cassette, 0.5mm spacers, and controlled by ALFwin 3.1 software. The data was processed by means of ALFwin 3.1 software, Amersham Pharmacia Biotech. The reaction set up, annealing temperature and PCR program is provided in the appendix.

Phylogenetic analysis:

The 16S rDNA sequence obtained was passed through the GenBank BLAST system and the results downloaded. A CLUSTAL W (Thompson *et al* 1994) input file was generated and a multiple sequence alignment was then performed. CLUSTAL W was then used to create a boot strp tree using 1000 iterations and this tree was then viewd in Tree View (Page 1996).

Results:

Only one species of bacteria was isolated from the uropygial gland of *P. purpureus*. This type strain was designated JLB-1. The cells are Gram positive cocci that group together in pairs or short chains. I observed neither motility nor endospore formation. On nutrient agar the strain JLB-1 forms small colonies of less than 1mm in diameter; these were regular and entire in shape and a grey-ish white colour. The bacterium is both catalase and oxidase negative and grows equally well aerobically or in the presence of 10% carbon dioxide. The bacterium grows under anaerobic conditions, but the growth is slower. I found that the bacterium was able to tolerate NaCl concentrations of between 0.5% and 3%. Similarly, the strain JLB-1 grew well between pH 6 and pH 9, with the best growth being at pH 8; it did not grow at pH 4.5. I did not observe hydrolysis of starch, gelatine, casein, urea, arginine, aesculin, Tween 80, hippurate or DNA. The bacterium was unable to use sodium citrate as its sole carbon source, did not produce indole, H₂S nor acetoin. No gas was produced during growth on any of the sugars; acid was produced from arabinose, cellobiose, cyclodextrine, fructose, glucose, maltose, mannose, rhamnose, ribose, salicin, sucrose, trehalose and xylose. I did not observe any acid production from galactose, inulin, melibiose, raffinose or sodium succinate. In the case of dulcitol, glycerol, inositol, lactose, mannitol, melezitose sorbitol, and sorbose, I only observed acid production after 48 hours of incubation. The bacterium did not grow on the M9 medium nor the M9 medium enriched with casitone, but I did observe a small amount of growth on the M9 medium enriched with yeast extract. This suggests that strain JLB-1 has a requirement for one or more growth factors. The results of the gas chromatography showed that strain JLB-1 produces lactic

acid and a minor amount of acetic acid, suggesting that it is an homofermentative, lactic acid bacterium. The biochemical and physiological characteristics distinguishing strain JLB-1 from other Gram positive lactic acid bacteria are represented in tables 3.1, 3.2 and 3.3.

PCR analysis:

The amplified 16S rDNA gene yielded a PCR product of 1472 bp. This almost complete gene sequence is presented in figure 3.1. This sequence was compared with

Figure 3.1: The partial 16S rDNA sequence of '*Enterococcus phoeniculicola*' as registered with GenBank (accession number AY028437).

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TTTCTAAACCCCTATACGGCTGGCTCCAAAAGGTTACCTCACCGGCTTCGGGTGTTTAC
AACTTTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGAACGTATTCACCGCGGCGTG
CTGATCCGCGATTACTAGCGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGA
ACTGAGAGAAGCTTTAAGAGATTAGCTTAGCCTTGCGACTTCGCGACTCGTTGTACTTCC
CATTGTAGCACGTGTGTAGCCAGGTCATAAGGGGCATGATGATTGACGTCATCCCCAC
CTTCCTCCGGTTTGTACCGGCAGTCTCGCTAGAGTGCCCAACTAAATGATGGCAACTAA
CAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGAC
AACCATGCACCACCTGTCACTTTGTCCCCGAAGGGAAAGTTCTGTCTCCAGAATGGTCAA
AGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACC
GCTTGTGCGGGCCCCCGTCAATTCCCTTTGAGTTTCAACCTTGCGGTTCGTAATCCCCAGGC
GGAGTGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCTCCAACACTTAGCACT
CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAG
CCTCAGCGTCAGTTACAGACCAGAGAGTCGCTTCGCCACTGGTGTTTCTCCATATATCT
ACGCATTTTACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCTCCCAGT
TTCCAATGACCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTG
CGCTCGCTTTACGCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTG
CTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGATACCGTCAAGGGAYGAACAGTTACTC
TCATCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGC
GGCGTTGCTCGGTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAG
GAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCTCTCAGGTGGGCTATGCAT
CGTGGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCACCGCGGGTCCATCCATCA
GTGACACCCGAAAGCGTCTTTCAAATTAGAACCATGCGGTTCCAATTGTTATACGGTATT
AGCACCTGTTTCCAAGTGTTATCCCCTTCTGATGGGCAGGTTACCCACGTGTTACTCACC
CGTCCGCCACTCTTTTTCTTTCGGTGGAGCAAGCTCCGGTGAAAGAAAAGCGTTCGACT
TGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGACCA
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other enterococci and streptococci, as well as a range of other lactic acid bacteria that were determined to be closely related by the GenBank system (*Carnobacterium*

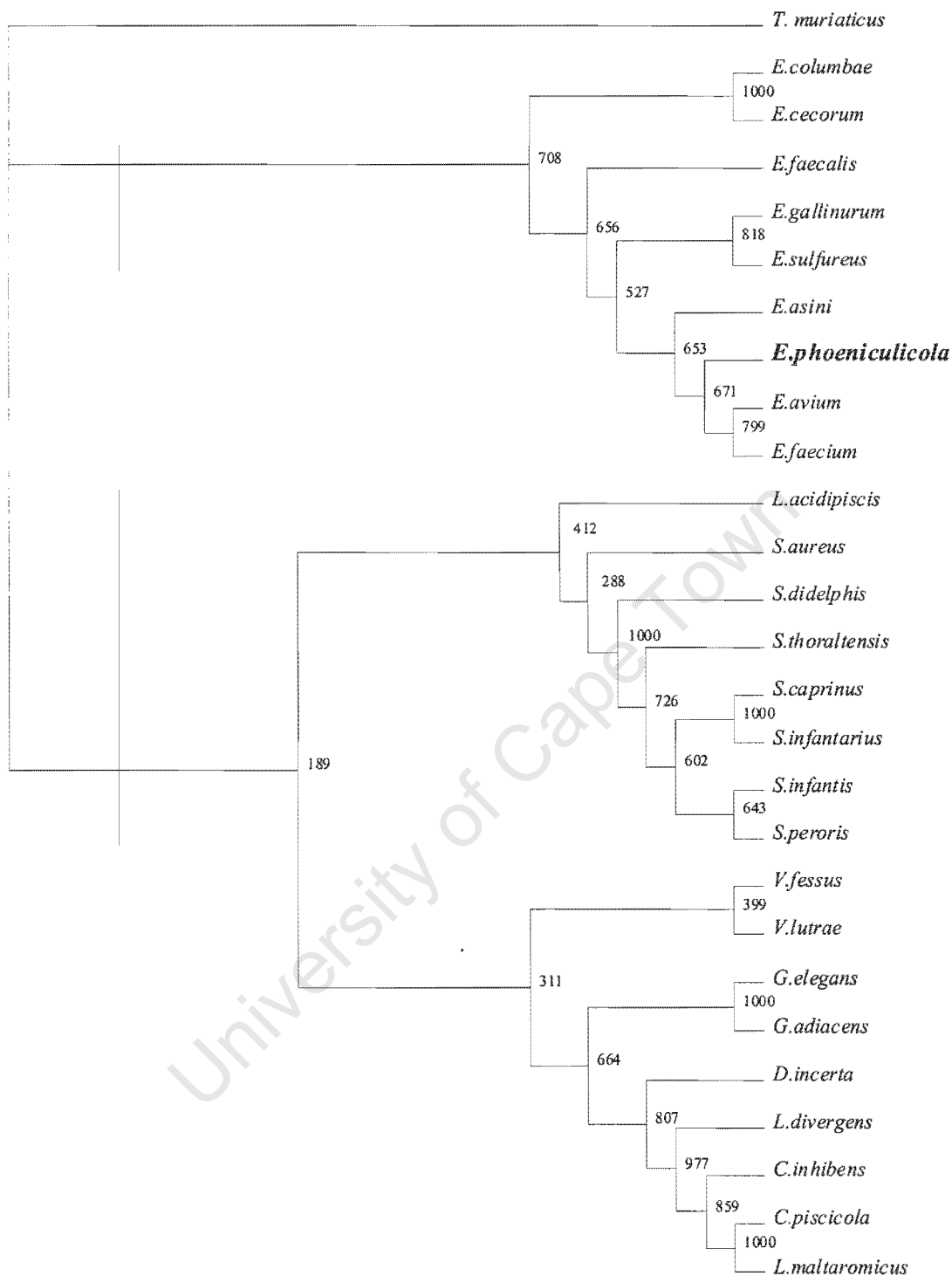


Figure 3.2: Unrooted phylogenetic tree based on 16S rDNA sequences, with *S. aureus* as an outgroup, showing the relationship between '*Enterococcus phoeniculicola*', enterococci, streptococci and other lactic acid bacteria. The tree was generated using the CLUSTAL W system and includes the bootstrap values generated.

Table 3.1: Physiological and biochemical characteristics that differentiate '*E. phoeniculicola*' from other enterococcal species.

	<i>E.</i> <i>phoeniculicola</i>	<i>E. faecium</i> [#]	<i>E. avium</i> [#]	<i>E. asini</i> [*]	<i>E. faecalis</i> [#]	<i>E.</i> <i>gallinarum</i> [#]	<i>E.</i> <i>sulfureus</i> [#]	<i>E.</i> <i>cecorum</i> ^{#,§}	<i>E.</i> <i>columbae</i> [#]
Acid from:									
Arabinose	+	+	+	-	+	+	-	-	-
Inulin	-	+	-	-	-	+	-	+	+
Lactose	-	+	+		+	+	+	+	+
Mannitol	-	+	+	-	+	+	-	-	+
Melezitose	-	-	+	-	+	-	+	+	+
Melibiose	-	+	+	-	-	+	+	+	+
Raffinose	-	+	-	-	-	+	+	+	+
Rhamnose	+	+	+	+	+	-	-	-	+
Ribose	+	+	+	-	+	+	+	+	+
Sorbitol	-	-	+	-	+	-	-	-	+
Sorbose	-	-	+	-	-	-		-	-
Sucrose	+	+	+		+	+	+	+	+
Xylose	+	+	+	+	+	+	+	-	+
Arginine:	-	+	+	+	+	+	-	-	-
Hippurate:	-	+	-	+	+	+	-	-	-
40% bile:	-	+	+	+	+	+		+	+
6% NaCl:	-	+	+	-	+	+	+	-	-
Opt. T (°C):	30 - 37	40	40	40	40	40	40	40	

*: de Vaux *et al* (1998); #: Carvalho *et al* (1998); §: Hsueh *et al* (2000)

Table 3.2: Physiological and biochemical characteristics that differentiate '*E. phoeniculicola*' from streptococcal species.

		<i>S.</i> <i>E. phoeniculicola</i> '	<i>S.</i> <i>didelphis</i> ^{\$}	<i>S.</i> <i>thoraltensis</i> [*]	<i>S.</i> <i>caprinus</i> [#]	<i>S.</i> <i>infantarius</i> [@]	<i>S.</i> <i>peroris</i> ^{&}	<i>S.</i> <i>infantis</i> ^{&}
Acid from:								
Arabinose	+	-	+	-	-	-	-	-
Inulin	-	-	+	-	-	-	-	-
Lactose	-	-	+	+	+	+	+	+
Maltose	+		+		+	+	+	+
Mannitol	-	-	+	+	-	-	-	-
Melibiose	-		variable			-	-	-
Raffinose	-	-	variable		variable	-	-	-
Ribose	+	+	+	-	-	-	-	-
Sorbitol	-		variable	-	-	-	-	-
Sucrose	+				+	+	positive	
Trehalose	+	+	+	+	-	-	-	-
Arginine:	-	+		-		-	-	-
Haemolysis:	none	+		-	alpha	+	+	+
V. P.:	-		+	+	+	-	-	-
Gelatin:	-					-	-	-
G + C:	51%		40%			39.8% - 40.5%	39.9% - 40.4%	

*: Devriese *et al* (1997); #: Sly *et al* (1997); @: Schlegel *et al* (2000); \$: Rurangirwa *et al* (2000); &: Kawamura *et al* (1998)

Table 3.3: Physiological and biochemical characteristics that differentiate '*E. phoeniculicola*' from other lactic acid bacterial species.

	<i>'E. phoeniculicola'</i>	<i>S. aureus</i> *	<i>L. acidipiscis</i> *	<i>D. incerta</i> *	<i>L. divergens</i> ☛	<i>C. inhibens</i> *	<i>C. piscicola</i> ▲
Acid from:							
Arabinose	+	-	variable	-	-	-	
Fructose	+	+	+	+	+	+	
Galactose	-	+	+	-	+	+	+
Glucose	+		+	+	+	+	+
Lactose	-	+		+	-	+	+
Maltose	+	+		+	+	+	+
Mannitol	-	+		-	-	+	+
Mannose	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	
Melibiose	-		-	+	-	-	+
Raffinose	-	-	-	-	-	-	+
Ribose	+	+	-	+	+	+	+
Sorbitol	-		-	-	-	-	-
Sucrose	+	+	variable		+	+	
Trehalose	+	+		+	+	+	+
Xylose	+	-	-		-	-	-
Arginine:	-	+	-	-		-	
Lactic acid:	+		+	L-lactic	+		
Spores:	-		-	-		-	-
Motility:	-		-	+		+	
Fac. Aer:	+	+	+	+			
Catalase:	-		-	-		-	-
Oxidase:	-			-		-	
Nit. Red.:	-	+	-			-	
Urease:	-	+		-			-

*: Stackebrand *et al* (1999); ▲: Coombs and Brenchley (1999); *: Tanasupawat *et al* (2000); *: Jöborn *et al* (1999); *: Kloos and Schleifer 1986; ☛: Kandler and Weiss 1986. For full names see page 27.

Table 3.3: Continued.

	<i>E. phoeniculicola</i>	<i>L. maltaromicus</i>	<i>G. elegans</i> ®	<i>G. adiacens</i> ®	<i>V. fessus</i> ⁺	<i>V. lutrae</i> [#]	<i>T. muriaticus</i> *
Acid from:							
Arabinose	+		-	-	-	-	-
Fructose	+						+
Galactose	-	+					
Glucose	+	+			+	+	
Lactose	-	+	-	-	-		-
Maltose	+	+				+	
Mannitol	-	+	-		-	-	+
Mannose	+	+					+
Melezitose	-		-	-	-	-	
Melibiose	-	+	-	-	-	-	
Raffinose	-	-	+	-	-	-	-
Ribose	+	+	-	-	-	+	
Sorbitol	-	-	-		-	+	-
Sucrose	+		+	+	-	+	
Trehalose	+	+	-	-	-	+	+
Xylose	+	-			-	-	
Arginine:	-		+	-	-	-	
Lactic acid:	+			+			
Spores:	-		-	-		-	
Motility:	-		-	-		+	-
Fac. Aer:	+		+	+	+	+	+
Catalase:	-	-	-	-	-	-	-
Oxidase:	-		-	-			-
Nit. Red.:	-				-	-	-
Urease:	-		-		-	-	

®: Collins and Lawson (2000); *: Satomi *et al* (1997); #: Lawson *et al* (1999); +: Hoyles *et al* (2000).

gallinarum (accession number: AJ387905), *C. inhibens* (Z73313), *C. piscicola* (AF184247), *Desemzia incerta* (Y17300), *Enterococcus asini* (Y11621), *E. avium*

(AF061008), *E. cecorum* (AF061009), *E. columbae* (AF061006), *E. faecalis* (AJ301831), *E. faecium* (AJ276355), *E. gallinarum* (AJ301833), *E. sulfureus* (AF061001), *Granulicatella adiacens* (AB022027), *G. elegans* (Y15413), *Lactobacillus acidipiscis* (AB023837), *L. divergens* (M58816), *L. maltaromicus* (M58825), *Streptococcus caprinus* (Y10869), *S. didelphis* (AF176108), *S. infantarius* (AF177729), *S. infantis* (AB008315), *S. peroris* (AB008314), *S. thoraltensis* (Y09007), *Tetragenococcus muriaticus* (D88824), *Vagococcus fessus* (AJ243326) and *V. lutrae* (Y17152) (figure 3.2). This comparison was performed using the NEIGHBOR and CONSENSE applications of the PHYLIP 3.5 system. The closest relatives of the unknown bacterium are *E. faecium* and *E. avium* (96 %).

G + C content:

The G + C content of the 16S rDNA of strain JLB-1 is 51%.

Discussion:

Strain JLB-1 is a Gram positive bacterium, with cells that are spherical or ovoid and forming pairs or short chains. By morphological appearance, Gram stain, endospore formation and motility, the unknown bacterium closely resembles the other members of the genus *Enterococcus*. The closest relatives of the strain JLB-1 are *E. avium* (97.53%) and *E. faecium* (97.34%). *E. avium* and *E. faecium* are positive for acid production from lactose, mannitol, melezitose and sorbitol while strain JLB-1 does not produce acid from these sugars. *E. avium* and *E. faecium* are also both able to hydrolyse arginine and grow in the presence of 40% bile and 6% NaCl while JLB-1 cannot (table 3.1). The ability to grow in NaCl concentrations of 6.5% or in 40% bile

was encompassed in the original definition of *Enterococcus* (Schleifer and Klipper-Bälz 1984). JLB-1 is homofermentative, producing lactic acid without producing gas; this follows the description of the enterococci as laid out in Bergey's Manual of Determinative Bacteriology (Holt *et al* 1994). The homology of the 16S rDNA sequence of JLB-1 and the enterococci is 96.00% (multiple alignment with *E. faecium*, *E. avium*, *E. asini*, *E. faecalis*, *E. gallinarum*, *E. sulfureus*, *E. cecorum* and *E. columbae*). The G + C content of the 16S rDNA of the bacterium is 51%, which is relatively high when compared with other enterococci, considering that *E. asini* has a genomic G + C content of only 39.4%.

As with the enterococci, the cell morphology, Gram stain and endospore properties of the streptococci are the same as strain JLB-1. Strain JLB-1 does not produce acid from melibiose, raffinose or sorbitol and is unable to hydrolyse gelatine. It does produce acid from sucrose. The streptococci are also homofermentative with the major product being lactic acid; strain JLB-1 is homofermentative, producing lactic acid and no gas. These are the only physiological and biochemical properties in which strain JLB-1 correlates with all the species of streptococci against which it was compared. Strain JLB-1 is not haemolytic whereas streptococci (lactic acid and virridans) are. Some species of streptococci show definite motility. Most species of streptococci are pathogenic, with their growth being enhanced by the presence of blood (Hardie 1984). The G + C content of the streptococci is also relatively lower than that of strain JLB-1, lying between 39% and 41% (table 3.2). The homology between the 16S rDNA sequence of JLB-1 and the streptococci is 86.66% (multiple alignment with *S. didelphis*, *S. thoraltensis*, *S. caprinus*, *S. infantarius*, *S. peroris* and *S. infantis*).

Granulicatella, recently removed from the genus *Abiotropha* (Collins and Lawson 2000) is closely related to *Aerococcus*, *Carnobacterium* and *Enterococcus*. Strain JLB-1 resembles species from *Granulicatella* in that the cells are Gram positive and form chains and pairs. They are also non-motile, do not form endospores, are facultatively anaerobic, catalase negative, oxidase negative, do not produce urease or indole, are homofermentative without producing gas. JLB-1, like *Granulicatella* spp., produce acid from sucrose and does not produce acid from mannitol, melibiose, melezitose, sorbitol, lactose, raffinose and inulin. It is also unable to hydrolyse arginine. However, the cells of *Granulicatella* spp. occur as single cells, as well as pairs and chains and they are pathogenic and haemolytic, whereas JLB-1 does not exhibit haemolysis. The 16S rDNA sequence homology between JLB-1 and members of the genus *Granulicatella* is 95.00% (multiple alignment with *G. elegans* and *G. adiacens*). All *Granulicatella* isolates are of human origin (Collins and Lawson 2000).

The tetragenococci resemble strain JLB-1 in that they are Gram positive, non-motile, do not produce endospores or catalase, and are homofermentative, producing lactic acid. The tetragenococci are also unable to hydrolyse arginine, grow optimally at pH 8, are facultatively anaerobic and are unable to reduce nitrate. Both produce acid from ribose, fructose, mannose, trehalose; neither produce acid from lactose, raffinose or sorbitol. However, the tetragenococci form tetrads when grown in broth and are unable to grow in the absence of NaCl. They grow in concentrations of NaCl between 1 and 25 % NaCl, with optimal growth occurring between 7 – 10 % NaCl. The 16S rDNA sequence homology between JLB-1 and *T. muriaticus* is 90.54%.

The genus *Desemzia* is also closely related to *Carnobacterium*. *Desemzia* resembles strain JLB-1 in that it is Gram positive, catalase and oxidase negative and produces lactic acid. It also produces acid from ribose and maltose, and does not produce acid from sorbitol or melezitose. It does not hydrolyse arginine or produce indole. It differs from JLB-1 in that the cells are rod shaped and occur singly. The 16S rDNA sequence homology between *D. incerta* and JLB-1 is 92.59%. It is a member of the *Bacillus* – *Lactobacillus* group, of the *Clostridium* – *Bacillus* sub-phylum.

The genus *Carnobacterium* resembles strain JLB-1 in that both are Gram positive, catalase negative and are unable to reduce nitrate. The cells of *Carnobacterium* are rod shaped, occurring singly or in pairs. They are heterofermentative, producing lactic acid; some members of the genus are known to be motile. The homology of the 16S rDNA gene sequence of JLB-1 and members of the genus *Carnobacterium* is 92.55% (multiple alignment with *C. inhibens* and *C. piscicola*).

The lactobacilli resemble strain JLB-1 in that both are Gram positive, do not form endospores, are facultatively anaerobic, do not reduce nitrate, are unable to hydrolyse gelatine or casein, do not produce H₂S, and are catalase and oxidase negative. However, the cells of the lactobacilli are rods that form short chains. Some members of *Lactobacillus* are motile and all are heterofermentative. The lactobacilli grow well between pH 5.5 and 6.2, with an optimal pH of 5.0 or less. The homology of the 16S rDNA sequences of JLB-1 and the lactobacilli is 90.10% (multiple alignment with *L. acidipiscis*, *L. divergens* and *L. maltaromicus*). Lactobacilli are found in a variety of food products and are used extensively in industry, they are isolated from the mouth, intestinal tract and vagina of humans (Kandler and Weiss 1984).

Figure 3.2 indicates that strain JLB-1 is more closely related to the enterococci and the streptococci than any of the other lactic acid bacteria. The phylogenetic tree supports the results of comparing the physiological and biochemical characteristics of strain JLB-1 with these other groups of lactic acid bacteria. The bootstrap values generated by PHYLIP indicate the relationships between the 16S rDNA of JLB-1 and 16S rDNA of other lactic acid bacteria. The smaller the bootstrap value, the less close the relationship between the bacterial groups; this echoes closely the comparison of the physiological and biochemical characteristics of the various groups of bacteria.

The fact that strain JLB-1 is catalase negative and homofermentative lead me to believe that it belonged to the lactic acid group of bacteria. I believe that this has been proven conclusively by the results of the physiological tests and the comparisons that have been drawn between JLB-1 and the lactic acid bacteria. JLB-1 shows the highest 16S rDNA sequence homology with the enterococci and for this reason I believe that it is a member of the genus *Enterococcus*. I isolated this bacterium from six individual adult Red-billed Woodhoopoes, *Phoeniculus purpureus*. It is unlikely that the bacteria have been cultured from the uropygial secretion of these birds before. The physiological, biochemical and genetic characteristics of this bacterium are sufficiently different from previously identified species of the *Enterococcus* genus that I propose a new species, '*Enterococcus phoeniculicola*'.

Description of *Enterococcus phoeniculicola* sp. nov.

Enterococcus phoeniculicola (phoe.nic.u.li.co'la. NL. masc. Adj. *phoeniculicola* growing in *Phoeniculus*, the genus of the woodhoopoe).

Cells are facultatively anaerobic, non-motile, non-sporing, catalase and oxidase negative, Gram positive cocci that occur as pairs or short chains. Colonies are whitish-grey, circular and ~ 0.5mm in diameter. Optimum growth occurs between 30°C and 37°C and at pH 8. Growth occurs in 3% NaCl, but not in 6% NaCl, 40% bile or 10% ethanol. Homofermentative, producing lactic acid and no gas (minor quantities of acetic acid were detected by gas chromatography). Positive for acid production from arabinose, cellobiose, cyclodextrine, fructose, glucose, maltose, mannose, rhamnose, ribose, salicin, sucrose, trehalose and xylose. No acid is produced from galactose, inulin, melibiose, raffinose, sodium succinate. Acid production is delayed (takes 48 hours) from dulcitol, glycerol, inositol, lactose, mannitol, sorbitol and sorbose. Does not liquefy gelatine, reduce nitrate, produce H₂S or acetoin, hydrolyse casein, Tween 80, starch, hippurate, DNA or arginine. No haemolysis on blood agar. Does not grow on M9 minimal media or M9 enriched with casitone, but grows on M9 enriched with yeast extract. The G + C content of the 16S rDNA is 51% (GenBank accession number of the 16S rDNA sequence: AY028437).

Habitat:

The type strain (JLB-1) was isolated from the uropygial gland of the Red-billed Woodhoopoe, *Phoeniculus purpureus*, captured in the Morgan Bay region of the Eastern Cape, South Africa (32° 43'S, 28° 47'E).

Appendix:

Acid from sugar:

Peptone water:

1% peptone

0.5% NaCl

distilled water

Place in capped test tubes and autoclave

To this add the required sugar solution, which has been filter sterilised, to a final concentration of 0.5%

To this add filter sterilised bromocresol purple to a concentration of 0.05g/l.

Hippurate hydrolysis:

1g tryptone

0.3g beef extract (Oxoid "Lab Lemco" powder)

0.1g yeast extract

0.5g Na₂HPO₄

1g Na hippurate

1.5g agar

100ml water

adjust to pH 7.0 and autoclave

Add 1ml 10% glucose

After inoculation and incubation, flood with 50% H₂SO₄

Look for the precipitation of crystals of benzoic acid for a positive reaction

(Gordon *et al* 1974)

Aesculin Hydrolysis:

0.3g yeast extract

0.05g ferric ammonium citrate

0.1g aesculin or arbutin

1.5g agar

100ml water

adjust to pH 7.0 and autoclave

Tween 80 hydrolysis: (Sierra's medium – lipase)

1g peptone (Oxoid #L34)

0.5g NaCl

0.012g $\text{CaCl}_3 \cdot 2\text{H}_2\text{O}$

1.5g agar

90ml water

Adjust to pH 7.4 and autoclave

1ml Tween 80

9ml water

Autoclave separately from agar

Allow to cool, mix and pour.

Arginine hydrolysis:

0.1g peptone

0.5g NaCl

0.03g K₂HPO₄

0.001g phenol red

1g L-arginine – HCL

1.5g agar

100ml H₂O

adjust to pH7.2

Place 10ml into MacCartney bottle and autoclave

Stab to bottom and seal with sterile liquid paraffin

A change in colour from orange to pink indicates that arginine has been hydrolysed to NH₃

Nitrate reduction:

Nutrient broth

0.2% potassium nitrate

0.6% bacteriological agar (Biolab Diagnostics[®])

Place in capped test tubes and autoclave.

Inoculate, incubate overnight at 37°C and add equal quantities of solutions A and B.

Solution A:

8g sulfanilic acid

1 litre 5M acetic acid

Solution B:

5g α – naphthylamine

1 litre 5M acetic acid

Methyl Red – Voges Proskauer test:

7g peptone

5g anhydrous K_2HPO_4

1 litre distilled water

5g glucose

Adjust to pH 7.5

Place in capped test tubes and autoclave

Inoculate, incubate for 48 hours and add either the Methyl – Red or Voges – Proskauer reagent

Methyl Red:

0.1g methyl red

300ml 96% ethanol

200ml distilled water

Voges – Proskauer:

10% KOH

Indole production:Peptone water:

1% peptone

0.5% NaCl

distilled water

Place in capped test tubes and autoclave

Inoculate and incubate for 48 hours

Add equal volumes of Ehrlich's reagent.

Ehrlich's reagent:

4g *para*-dimethyl-amido-benzaldehyde

380ml absolute ethanol

80ml concentrated HCl

M9 minimal media:

6g Na₂HPO₄

3g KH₂PO₄

0.5g NaCl

1g NH₄Cl

15g agar

1 litre distilled water

adjust to pH 7.4, autoclave, cool and add

2ml 1M MgSO₄

10ml 20% glucose

0.1ml 1M CaCl_2

sterilize above separately by autoclaving or filtration (glucose)

Acid production:

Volatile fatty acids:

1.0ml sample

0.2ml H_2SO_4

0.4g NaCl

1.0ml diethyl ether

add to centrifuge tube and vortex

centrifuge at 3000 rpm for 3min

remove upper ether layer and place in screw top bottle

add anhydrous MgSO_4 (kept in hot air oven) to ether layer

leave for 5min, transfer to AS vial and cap immediately

Non – volatile acids:

1.0ml sample

0.4ml 50% H_2SO_4

3.0ml methanol

use conical centrifuge tube if possible

vortex

Place in heated dry box at 60°C for 30 – 40min

Vortex and centrifuge at 3000 rpm for 3min

PCR amplification:

2µl DNA

2µl MgCl₂ (25mM)

5µl 10 x buffer

0.5µl Taq polymerase (Super-Therm JMR – 801)

3.5µl F₁ primer (10µM)

3.5µl R₅ primer (10µM)

0.5µl dNTPs (25mM)

35µl water

PCR program: (Biometra[®] Personal Cycler)

Step 1: 96°C for 120 seconds

Step 2: 96°C for 45 seconds

Step 3: 58°C for 30 seconds

Step 4: 72°C for 90 seconds

Repeat steps 2,3 and 4 thirty times

Step 5: 72°C for 300 seconds

Step 6: 4°C hold

IV. Woodhoopoe defence: Pathogens & parasites

Summary:

Red-billed Woodhoopoes, *Phoeniculus purpureus*, produce a secretion from their uropygial gland that consists of at least 17 compounds. The uropygial secretion produced by *P. purpureus* was artificially synthesized and the individual compounds found within the secretion diluted to the concentration found in the natural secretion. These were tested, by means of disc-diffusion assays, against 13 species of pathogenic bacteria and one parasitic bacterium. The secretion was tested in quantities similar to what is expected that the birds would apply to their own feathers. Seven of the compounds found within the secretion, as well as the synthetic secretion, showed inhibitory action against these bacterial species. This suggests that the secretion is likely to be a potent inhibitor of disease and feather degradation on the birds. The synthetic secretion was also tested against feather mites on a family of six birds. The mites did not show any significant reaction to the secretion.

Introduction:

All organisms co-exist with symbionts; be they commensals, mutuals or parasites. Bacteria are found in all habitats and occur as normal flora on, and within, all organisms. Many normal flora have the ability to become opportunistic pathogens in the young (Burt 1999) and those whose immunocompetence is lowered (Prescott *et al* 1993). Many species of bacteria have been identified from nest sites and cloacal samples of birds, included in these are *Pseudomonas sp.*, *Bacillus sp.*, *Escherichia sp.*, *Staphylococcus sp.*, which are normal flora of birds, as well as *Salmonella sp.* and *Shigella sp.*, which are pathogenic (Cooper 1990, Cooper 1993, Hubálek *et al* 1995, Singleton and Harper 1998, Burt 1999). Birds may be exposed to pathogenic bacteria by a variety of means: 1) exposure to conspecifics which may be diseased or healthy carriers, 2) roosting or nesting in previously used cavities, 3) food items that are

contaminated, and 4) parasites, such as ticks, which may act as vectors for disease (Hubálek *et al* 1995).

Bacteria and fungi may also be parasitic. Burt and Ichida (1999) isolated *Bacillus licheniformis* and *B. pumilus* from wild caught birds and showed that both of these species actively degrade feathers, while Hubálek (1976) isolated numerous species of keratinolytic fungi from wild birds occurring in Czechoslovakia and Yugoslavia. Microbes such as these would normally be found in soil or water (Hubálek 1976, Burt and Ichida 1999) where they would degrade feathers that had been dropped or moulted. Birds that are constantly exposed to areas in which such microbes are found, continually run the risk of contaminating their feather with such organisms. The latter may be defined as parasites as their degradation of feathers poses a significant risk to birds due to a loss of insulation and lowering the flight ability of birds (Clayton 1991, Harper 1999).

Most birds carry small numbers of arthropod ectoparasites. Such parasites may be controlled by preening or scratching, with bills and feet (Rózsa 1993, Clayton 1991). Birds which are deformed in some way, such as damage to the bill or feet (Clayton 1991) show an exponential increase in arthropod ectoparasites. Birds with long unwieldy bills tend to scratch more as their preening abilities are not as good as their short-billed counterparts, they may also participate in allopreening which allows individuals to remove parasites from other individuals. Parasites can cause extensive damage to feathers and therefore birds' insulative and flight capabilities. Clayton (1990) showed that feather weight / density is inversely proportional to parasite numbers. Birds who have high parasite numbers also tend to have duller plumage after

moult as well as shorter primaries (Harper 1999). Arthropod ectoparasites not only damage feathers but also cause anaemia, nestling mortality and act as vectors for other parasites and pathogens (Dobson and Hudson 1986, Threlfall 1986). Parasites that damage feathers and depress the immune system lessen the probability of an individual mating successfully (Clayton 1990, Clayton 1991b).

Red-billed woodhoopoes, *Phoeniculus purpureus*, commonly occur in the mesic woodlands in the eastern parts of Africa. These birds have a large uropygial gland from which they secrete a microbially modified secretion consisting of at least 17 compounds (refer to chapter 2). When threatened by a predator within the roost site, the birds present their uropygial gland towards the opening of the roost site and the source of the threat. The birds roost communally in cavities and breed cooperatively. Groups range in size from two to 12 members and the group is comprised of a single breeding pair and a number of non-breeding helpers (Ligon and Ligon 1978, Du Plessis 1993). Woodhoopoe territories are characterized by having a number of roost sites within the territory (Du Plessis 1992). Cavity roosting helps the birds maintain body temperature and thereby increases their chances of survival during times of extreme cold (Du Plessis and Williams 1994). Cavity roosting also increases the chances of vertical and horizontal transmission of pathogens and parasites within the family group.

To date few studies have focussed on how birds may defend themselves against pathogens and parasites. This study was aimed at testing whether the chemical compounds secreted by the uropygial gland of the Red-billed Woodhoopoe have any effect on microbial and arthropod associates of the birds. Specifically, are the

woodhoopoes capable of lowering their susceptibility to disease and bodily harm by microbial and arthropod parasites?

Methods:

A quantity of the *P. purpureus* secretion was artificially synthesised by the Laboratory for Ecological Chemistry at the University of Stellenbosch – this synthetic secretion mimicked the secretion in birds caught in the Morgan Bay area of the Eastern Cape (32° 43'S, 28° 47'E). Quantities of the individual constituent components of the secretion were also obtained from the same source.

Bacterial associates:

Cultures of *Salmonella gallinarum*, *Staphylococcus aureus*, *Bordetella avium*, *Pseudomonas aeruginosa*, *Streptococcus zooepidemicus*, *Pasteurella multocida*, *Escherichia coli*, *Streptococcus faecalis*, *Clostridium perfringens*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Pasteurella haemolytica* and *Pasteurella gallinarum* were obtained from Allerton Provincial Veterinary Laboratory in Pietermaritzburg, KwaZulu - Natal. The bacterial species chosen are known avian pathogens. The culture of *Bacillus licheniformis* was obtained from the Department of Molecular and Cell Biology, University of Cape Town. Table 4.1 summarizes the effects of these bacteria, as well as their oxygen requirements and the temperature at which they were cultured. Those species that required 10% CO₂ were cultured in an anaerobic jar to which 10% CO₂ had been added; *C. perfringens* was cultured in an anaerobic hood. These were maintained using Brain-Heart Infusion broth

Table 4.1: The 14 bacterial species against which the uropygial secretion, and its constituent compounds, of the Rebbled Woodhoopoe were tested for anyibiotic properties. Also indicated are the effects of the bacteria, the temperature at which they were cultured, their preferred oxygen level and their cell wall type, according to Bergey's Manual (in Prescott *et al* 1993).

<u>Scientific name:</u>	<u>Disease:</u>	<u>Culture Temperature:</u>	<u>Culture conditions:</u>	<u>Cell wall type:</u>
<i>Escherichia coli</i>	colisepticaemia	37°C	aerobic	negative
<i>Bordetella avium</i>	respiratory infection	37°C	10% CO2	negative
<i>Salmonella gallinarum</i>	generalised infection	37°C	aerobic	negative
<i>Salmonella typhimurium</i>	generalised infection	37°C	aerobic	negative
<i>Salmonella enteritidis</i>	generalised infection	37°C	aerobic	negative
<i>Psuedomonas aeruginosa</i>	respiratory/ generalised	37°C	aerobic	negative
<i>Pasteurella multocida</i>	respiratory / generalised	37°C	10% CO2	negative
<i>Pasteurella haemolytica</i>	respiratory / generalised	37°C	10% CO2	negative
<i>Pasteurella gallinarum</i>	respiratory / generalised	37°C	10% CO2	negative
<i>Staphylococcus aureus</i>	joints / skin / generalised	37°C	aerobic	positive
<i>Streptococcus faecalis</i>	generalised infection	37°C	aerobic	positive
<i>Streptococcus zooepidemicus</i>	generalised infection	37°C	aerobic	positive
<i>Clostridium perfringens</i>	gas gangrene	37°C	anaerobic	positive
<i>Bacillus licheniformis</i>	feather degradation	37°C	aerobic	positive

(Merck). The bacteria were cultured overnight in Brain-Heart Infusion broth. The optical densities (OD) of these cultures were measured at 400nm; the cultures were then diluted using sterile Brain-Heart Infusion broth to a uniform OD of 0.1. These diluted

cultures were then used when performing the disc-diffusion assays. The synthetic secretion and its constituent compounds were tested against these diseases by means of disc-diffusion assay. The assays were cultured on Brain-Heart Infusion agar using sterile 6mm diameter discs of Whatman[®] No. 1 filter paper onto which 10µl of the various solutions had been pipetted. Brain-Heart Infusion agar was formed using Brain-Heart Infusion broth and adding Bacteriological agar (Biolab) up to a concentration of 1.5%. The chemical constituents of the secretion were diluted to the concentration in which they are found in the natural secretion. Those which were water-soluble were diluted in water and those which were not water soluble were diluted in 100% dimethylsulfoxide (DMSO) (refer to Table 2.1). DMSO was chosen as the chemical solvent as, even at a concentration of 100%, it did not have any inhibitory effect on the bacteria. This was tested by means of pipetting 10µl of DMSO and 10µl of sterile water onto discs of Whatman filter paper and placing them on plates onto which 100µl of the bacterial broth cultures had been pipetted and spread. The artificial secretion was tested against the different species of bacteria in the same manner. The cultures were incubated at 37 C overnight according to the oxygen requirements of each bacterium and the diameters of the zones of inhibition were then measured diagonally across the zone using a clearly marked 15 cm ruler.

Arthropod associates:

A family group of six Red-billed Woodhoopoes; three females and three males; were held in captivity in the aviaries of the University of Cape Town. The family group was held in one aviary and a single individual was separated (placed in an adjacent aviary) from the group for a period of 48 hours, to prevent allopreening or horizontal transmission of parasites. A small quantity of the artificial secretion was applied to the

third primary of the left wing, of the separated individual, while a similar quantity of sterile saline was applied to the third primary of the right wing. The total number of parasites on both primaries was counted using a magnifying glass before the application of the secretion and saline, and again after the 48 hours. This process was repeated with all the members of the family group. The results of the tests of the effect of the secretion upon arthropod ectoparasites were analysed using the Wilcoxon Matched Pairs test (Zar1996).

Samples of the parasites found on the woodhoopoes were collected from birds in the Morgan Bay area of the Eastern Cape. This was done by dusting the birds with Karbo-Dust[®] and allowing the birds to “shake” themselves in a paper packet. These samples were collected and the parasites removed and preserved in alcohol. The samples were then sent to ARC – Plant Protection Research Institute, Pretoria, for identification.

Results:

Bacterial associates:

The secretion produced by *P. purpureus* contains at least 17 compounds; of these only seven showed any inhibitory effect against the 14 bacterial species against which they were tested. Figure 4.1 shows the sizes of the inhibition zones produced by the full secretion and those chemical constituents of the secretion that showed any activity against the bacteria. There was substantial variation in the sizes of the inhibition zones produced both within the chemical groups and among the bacterial species themselves.

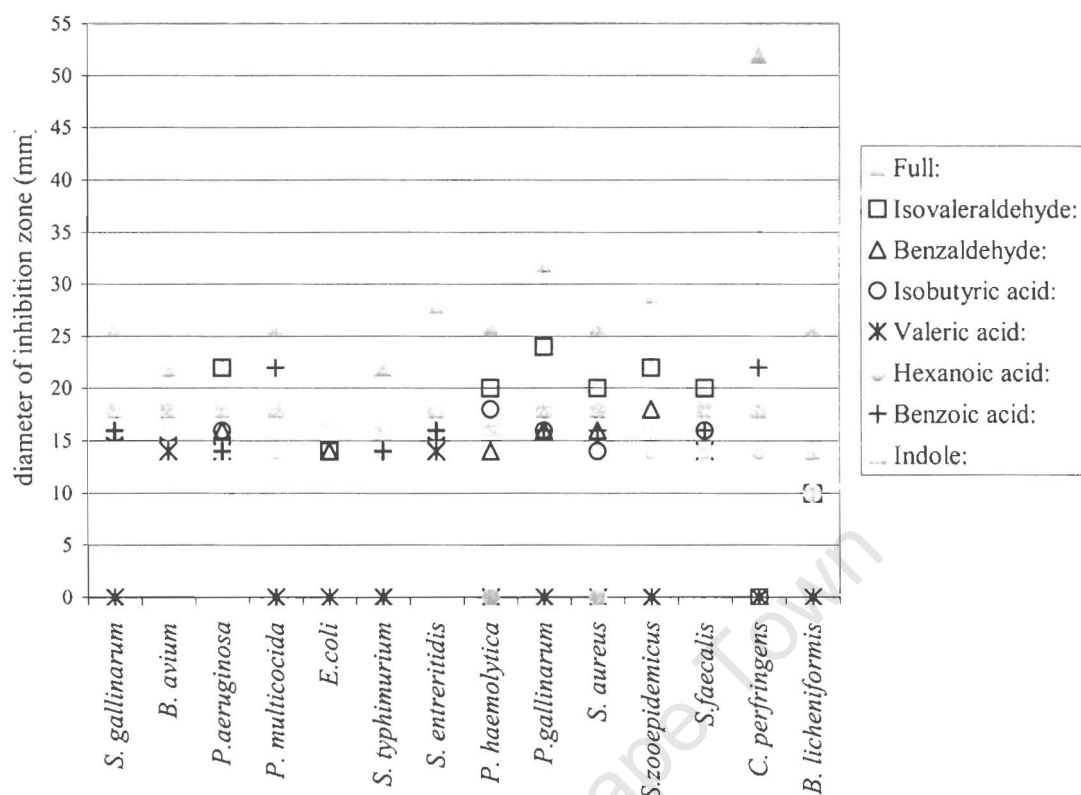


Figure 4.1: The diameters of the inhibition zones, in millimetres, produced by the full synthetic secretion and its constituent compounds, when tested against the pathogenic bacterial species. The inhibition zones include the diameter of the filter paper discs. The chemical constituents were arranged according to chemical groups and the bacterial species, according to cell wall type. Propionaldehyde, heptaldehyde, propionic acid, γ -butyrolactone, σ -valerolactam, hexanal, dimethyl disulfide, phenol and trimethylamine did not show any inhibitory effect against the bacterial species.

In the case of *C. perfringens*, the compounds within the full secretion had a synergistic effect against the bacteria. As a group the acids appear to have the greatest inhibitory effect upon the bacterial species, followed by the aldehydes, with indole being the only chemical from the ‘miscellaneous’ group having any effect upon the bacteria. Benzoic

acid, as found in the secretion, is highly concentrated, which accounts for the relatively large inhibition zones produced by this acid against some of the bacterial species.

Table 4.2: The number of ectoparasites found on the third primary of the left and right wings of the six Red-billed Woodhoopoes before and after the application of the synthetic secretion and sterile saline respectively. None of the comparisons were significant (Wilcoxon matched pairs test (Zar 1996)).

<u>Date:</u>	<u>Bird:</u>	<u>Left before secretion:</u>	<u>Left after secretion:</u>	<u>Right before saline:</u>	<u>Right after saline:</u>
24/09/99	DB	2	2	0	5
26/09/99	LG	5	2	4	0
28/09/99	DG	6	5	5	6
30/09/99	R	2	4	1	10
02/10/99	Y	7	6	10	12
04/10/99	O	2	5	4	3

Arthropod associates:

Two species of feather mites, from the wild-caught *P. purpureus*, were recorded and identified as *Uniscutalges phoeniculi* and *Pteronyssoides bouweri* respectively. Parasite numbers on the third primary of the left wing were compared before and after the application of the synthetic secretion ($Z = 0.000$, $p = 1.000$). Similarly, this was done for the third primary of the right wing before and after the application of the sterile saline ($Z = 1.048$, $p = 0.295$). The numbers of parasites for the third primary of the left and right wings were also compared before ($Z = 0.000$, $p = 1.000$) and after ($Z = 1.153$, $p = 0.249$) the application of the synthetic secretion and sterile saline respectively. The differences in number of parasites before and after the application of the synthetic

secretion and the sterile saline were also compared ($Z = 0.941$, $p = 0.347$). There was no pattern in the movement of the mites with regard to the secretion, with mite numbers increasing and decreasing randomly for both the secretion and the saline treatments.

Discussion:

Pathogens:

Not all of the compounds had an inhibitory effect upon the bacteria. There is no obvious pattern to the size of the inhibition zones with regard to chemical group, cell wall type or oxygen requirements of the bacteria. The amount of each compound, and the full synthetic secretion, was relatively small. However, this amount is representative of the amount that is spread across the feathers by the birds' bill. This shows that the secretion is a potent inhibitor of bacteria that may be living within the birds' feathers.

There appears to be no correlation between the cell wall type of the bacterial species and the size of the inhibition zone produced by the compounds or the full secretion. In the case of *Clostridium perfringens* the full secretion seems to show a synergistic effect of the combined constituents of the secretion, while some of those individual constituents had relatively little effect on the bacterium. This also appears to be the case with *S. gallinarum*, *S. aureus*, *S. zooepidemicus*, *P. multocida*, *S. enteritidis*, *P. haemolytica* and *P. gallinarum*. However, in the case of *E. coli*, some of the constituent compounds had a greater effect upon the bacterium than did the full secretion. *E. coli* is part of the normal gut flora of the bird (Cooper *et al* 1986) and is therefore likely to have developed a resistance to the secretion through ingestion. It is unlikely that the birds would have ingested the compounds of the secretion individually with any regularity.

This would explain the fact that *E. coli* shows a higher susceptibility to the individual compounds than to the entire secretion.

Birds do ingest their uropygial secretion (Elder 1954) and, in the case of woodhoopoes, this will afford protection against ingested bacteria that may be opportunistic or obligate pathogens. Wiping the bill over the body, or scratching an irritated area with the tip of the bill could conceivably bring the secretion into contact with infections, thereby disinfecting the wound.

Parasites:

Bacillus licheniformis may be seen as a parasitic bacterium as it has a slower action than the pathogenic bacteria mentioned, however this action may also be potentially lethal. High parasite numbers may cause the degradation of feathers (Clayton 1991, Burt and Ichida 1999), which lowers the insulative ability of the feathers (Clayton 1991) and decreases the fitness and flight ability of birds by distorting feather growth after moult (Harper 1999, Muza *et al* 2000). *B. licheniformis* shows a higher susceptibility to the full secretion than any of the pathogenic bacteria tested. The uropygial secretion of *P. purpureus* therefore not only protects and waterproofs feathers (Livezey *et al* 1986) but also lowers the growth of degradative bacteria on the feathers. This will help boost the fitness, flight ability and thermoregulation of the birds.

Symbionts:

The secretion does not apparently provide the birds with any protection against arthropod ectoparasite invasion. This is not the case with homobatrachotoxin in the *Pitohui*. Dumbacher (1999) showed that lice preferred feeding on non-toxic feathers and that the toxin in feathers decreased the life span and activity levels of these lice.

The synthetic woodhoopoe secretion was tested only on feather mites of the woodhoopoes and not on lice, ticks, fleas and flies, which provides an extra avenue for research. Feather mites have not been shown to damage birds or their feathers in any way, hence the birds may not need to protect themselves against these arthropods. Mites are relatively host specific (Clayton 1991), evolving along with the bird, and, in the case of the woodhoopoe, the uropygial secretion produced by the bird. This co-evolution may provide an extra clue to the apparent lack of effect of the secretion on mites. Alternatively, some arthropod ectoparasites are not found permanently residing on birds. Indeed, many of these parasites live within the nest or roost cavities and only come into contact with birds when they birds are in residence within these cavities (Threlfall 1986). Woodhoopoes have a number of roost sites within their territories (Du Plessis 1992) which is possibly a further defence against infestation of parasites and pathogens.

Pathogens and parasitic bacteria will flourish in a cavity roost or nest environment as the roost is warmed by a number of bodies and the humidity is stable; both of which are conducive to rapid bacterial growth. The uropygial secretion of the Red-billed Woodhoopoe protects it from circumstances that it cannot avoid due to the physiological constraints of cavity roosting, as well as its social nature.

V. Woodhoopoe Defence: Predators.

Summary:

The Red-billed Woodhoopoe, *Phoeniculus purpureus*, produces a microbially modified secretion from its uropygial gland. This was analysed chemically and an artificial quantity of this synthesised. The secretion consists of six major chemical groups represented by approximately 17 compounds. This secretion causes an aversive reaction in cats when they are presented with food tainted with this secretion. The palatability of five of the constituent compounds, dimethyldisulfide (DDS), indole, phenol, propionaldehyde, propanoic acid, representing five of the six the major groups, were tested on naive domestic cats in an attempt to determine which of the groups of compounds caused this reaction. Indole, phenol and propionaldehyde significantly alter the palatability of food to cats. The effect of the full synthetic secretion (all 17 constituent compounds) was also tested on Rock Monitor Lizards to determine whether or not they show a similar reaction to the secretion as mammalian predators. The secretion was highly unpalatable to the Monitors and significantly reduced the number of mice eaten by the lizards. I conclude that the secretion has an anti-predatory defence function that deters both mammalian and reptilian predators.

Introduction:

The avoidance of predation is central to the survival and success of all species. Mechanisms of predator avoidance which have evolved in birds include crypsis and gregariousness; less well documented is the evolution of toxicity and unpalatability (Dumbacher and Pruett-Jones 1996). Aposematic colouration of animals serves as a warning to predators of potentially unpalatable or toxic prey (Edmunds 1974).

Red-billed Woodhoopoes, *Phoeniculus purpureus*, are brightly coloured, highly conspicuous, group-living birds (Ligon and Ligon 1978b, Du Plessis 1993). Woodhoopoes produce a strongly scented secretion from the uropygial gland situated

just above the base of the tail. In times of threat, particularly within the roost, the birds point this gland at the threat (Ligon and Ligon 1978b) and a drop of the secretion is formed at tip of the papilla of the uropygial gland. Woodhoopoe nest cavities and roost sites carry the distinctive odour of their residents (Steyn 1996).

Predation occurs across the entire spectrum of life. Mammals and reptiles are well documented predators of birds (Cott 1953, Pitman 1962, Pitman 1962b, van Aarde 1980, Catling 1988, Dyer 1996), preying not only upon adults but also on nestlings and eggs. Typical mammalian raiders of Red-billed Woodhoopoe roost cavities across their range include nocturnal, arboreal predators such as genets, *Genetta* spp., tree civets, *Nandinia binotata*, African wild cats, *Felis lybica*, and feral domestic cats, *Felis sylvestrus* (Ligon and Ligon 1978). Reptilian predators which will readily take eggs, nestlings, fledglings and adults, include the Spotted Skaapsteker, *Psammophylax rhombeatus*, Boomslang, *Dispholidus typhus*, Common Egg-eater, *Dasypeltis scabra*, Cape Cobra, *Naja nivea*, the mambas, *Dendroaspis* spp., and the Monitor Lizards, *Varanus* spp (Pitman 1962, Pitman 1962b, Branch 1998).

Wanless (1998) showed that the full secretion deters domestic cats from eating food tainted with it. In this study, I attempt to determine which of the constituent groups of compounds are active in the aversive reaction observed in domestic cats. I also investigate whether or not the full secretion causes a similar aversive reaction in reptilian predators, namely Rock Monitor Lizards.

Methods:

Red-billed Woodhoopoes, *Phoeniculus purpureus*, commonly occur in the mesic woodlands in the eastern parts of Africa. These birds have a large uropygial gland from which they secrete a microbially modified secretion consisting of at least 17 compounds (see chapter 2). When threatened by a predator within the roost site, the birds present their uropygial gland towards the opening of the roost site and the source of the threat. The birds obligately roost communally in cavities and breed cooperatively (Ligon and Ligon 1978, Du Plessis and Williams 1994). Groups size ranges from two to 12 members and the group is comprised of a single breeding pair and a number of non-breeding helpers (Ligon and Ligon 1978b, Du Plessis 1993). Woodhoopoe territories are characterized by having a number of roost sites within the territory (Du Plessis 1992). Woodhoopoes are depredated by mammals, birds and reptiles. Diurnally raptors of the *Accipiter* species hunt free-flying woodhoopoes as they forage. Additionally, their nests are visited by arboreal snakes and Monitor Lizards. Nocturnally, the roosts and nests of the woodhoopoes are vulnerable to predation by arboreal mammals, including genets and cats.

Mammalian predators:

Cafeteria experiments were carried out on healthy, naïve adult domestic cats at the Society for the Prevention of Cruelty to Animals (SPCA). The cats had been denied food for 16 hours prior to the experiment and were isolated from other cats, and humans, during the experimental process. The cats were presented with a choice of two bowls containing $20\text{g} \pm 0.3\text{g}$ of Pamper[®] Chicken and Beef, a commercial cat food

readily available at most supermarkets. Dimethyldisulfide (DDS), indole, phenol, propionaldehyde, propanoic acid and trimethylamine were chosen as representatives of the six major groups of chemical compounds found within the secretion. However, due to the fact that Trimethylamine is a gas, it was not used for the purposes of the cafeteria experiments. One of the bowls of food was laced with four drops of one of the five constituent compounds of the secretion while the other was laced with four drops of the corresponding solvent. The cats were allowed to eat until they no longer displayed any interest in either of the food bowls. Only then were they removed from the experimental enclosure and returned to their living enclosure and provided with food *ad libitum*. The bowls were then re-weighed to determine the mass of food eaten and the results compared using the Wilcoxon Paired Sample Test (Zar 1996). All of the cats ate of the food offered to them after the experiment and showed no ill effects of eating the food laced with the chemical constituents of the secretion or the corresponding solvents. The cats were not traumatised at any stage during these experiments.

Reptilian predators:

Similar cafeteria experiments were carried out using Rock Monitor lizards, *Varanus albigularis*, housed at the Worcester Snake Park and Die Vonds Reptile Centre, Paarl. The Rock Monitors were offered a choice of freshly killed mice, one of which had been laced with three drops of the synthetic woodhoopoe secretion, the other with the same volume of sterile saline. The mice were placed approximately 20 cm apart and approximately 20cm away from the monitor's head. The monitors were observed until such time as they actively retreated more than one meter away from the mice, after which the uneaten mice were removed from the enclosure. I recorded whether the eaten mice had been treated with the synthetic woodhoopoe secretion or sterile saline and

analysed the results using Fisher's Exact Test (Zar 1996). The monitors are usually only fed every second day, and provided with three or four mice per meal, but in this case they had been denied food for three days prior to the experiment. The ambient temperature was between 22°C and 25°C, high enough to ensure that the lizards would eat. The monitor lizards showed no ill effects of having eaten the mice provided.

To avoid pseudoreplication the same animals were never used more than once for this experiment. Due to the difficulty of obtaining a large enough sample size and the correct conditions for conducting the experiments, experiments could not be run to determine the effect of the individual groups of compounds upon the Rock Monitor Lizards.

Results:

Wanless (1998) carried out preliminary tests of the full synthesized Woodhoopoe secretion against domestic cats housed at the SPCA. His results showed that the secretion had a highly significant effect (Wilcoxon Paired Sample Test: $p = 0.0001$) on how much of the synthetically-treated food the cats would eat (Figure 5.1). Following this work, I investigated which of the groups of compounds found within the secretion were causing the aversion in the cats.

Mammalian predators:

There was no difference in the amount of food consumed by the cats when the food was laced with DDS or its corresponding solvent, dimethylsulfoxide (DMSO) (Wilcoxon Matched Pairs Test: $Z = 1.478$, $p = 0.140$), neither was there a difference between propionic acid and its solvent, water (Wilcoxon Matched Pairs Test: $Z = 0.968$,

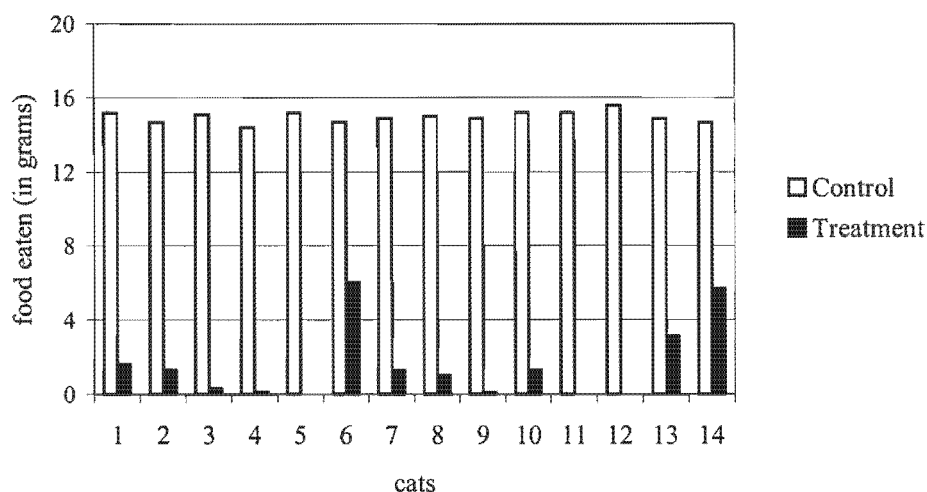


Figure 5.1: Wanless' (1998) offered cats food laced with drops of the full synthetic secretion and food not laced with any compound. The graph shows the disparity between the amounts of food eaten by the cats.

$p = 0.333$). There was, however, a significant difference in the quantity of food eaten by the cats when the food was laced with Propionaldehyde or water (Wilcoxon Matched Pairs Test: $Z = 2.701$, $p = 0.007$), Phenol or water (Wilcoxon Matched Pairs Test: $Z = 2.497$, $p = 0.013$) and Indole or DMSO (Wilcoxon Matched Pairs Test: $Z = 2.803$, $p = 0.005$) (Figure 5.2).

In some instances the bowl of food which was first approached by the cats was not the first bowl from which they ate (Table 5.1). DDS has a strong sulphurous smell, which the cats apparently found offensive. The cats would continuously move between the bowls of food, preferentially eating of the food laced with DMSO. However, when the bowl of control food was empty they would return to the bowl containing the food laced with DDS and slowly consume what remained in the bowl. It took the cats appreciably

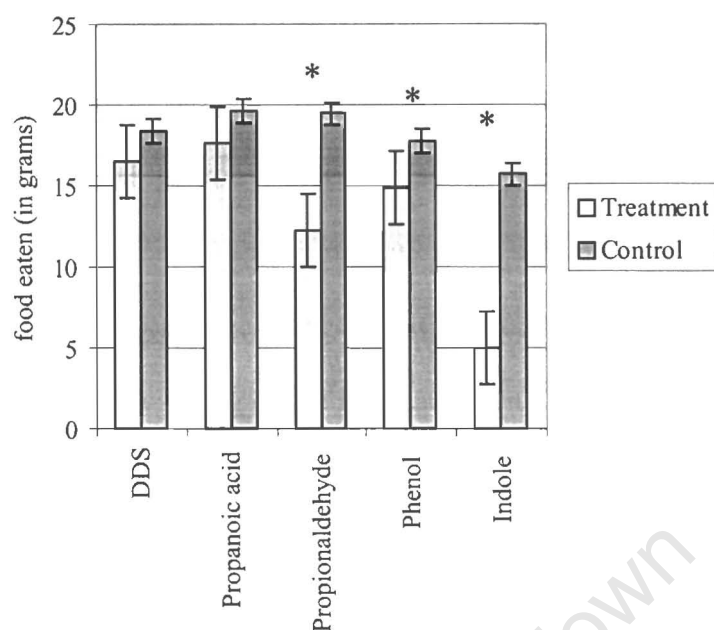


Figure 5.2: The quantity of food consumed by the cats when presented with food laced with the above compounds or their corresponding solvents.

* $p < 0.05$ (Wilcoxon matched pairs test (Zar1996)).

DDS = dimethyldisulfide

longer to eat the food laced with DDS than food laced with any of the other chemicals (Figure 5.3).

The difference in time taken to consume the food treated with the synthetic secretion as opposed to the time taken to consume the food treated with sterile saline was significant in all the tests (Wilcoxon Matched Pairs Test: DDS: $Z = 2.803$, $p = 0.005$; Propanoic acid: $Z = 2.479$, $p = 0.013$; Propionaldehyde: $Z = 2.803$, $p = 0.005$; Phenol: $Z = 2.803$, $p = 0.005$; Indole: $Z = 2.803$, $p = 0.005$). Although the cats consumed significantly less

of the treatment than the control food in the cases of indole, phenol and propionaldehyde,

Table 5.1: The first approaches and first choices of the food bowls, made by the cats when they were presented with the choice of treatment (T) and control (C) foods. Although, in many instances the treatment food was approached first, the cats consumed the control food before the treatment food.

	<u>DDS</u>		<u>Propanoic</u>		<u>Propion- aldehyde*</u>		<u>Phenol*</u>		<u>Indole*</u>	
	<u>First approach</u>	<u>First choice</u>	<u>First approach</u>	<u>First choice</u>	<u>First approach</u>	<u>First choice</u>	<u>First approach</u>	<u>First choice</u>	<u>First approach</u>	<u>First choice</u>
1	T	C	C	C	T	T	C	C	T	T
2	T	C	C	C	T	C	T	T	C	C
3	T	C	T	C	T	C	T	C	T	C
4	C	C	C	C	T	C	T	C	T	C
5	T	C	T	T	C	C	C	C	C	C
6	C	C	C	C	T	T	T	C	C	C
7	C	C	T	T	C	C	T	C	C	C
8	C	C	T	C	C	C	C	C	T	C
9	T	C	T	C	T	C	C	C	T	C
10	C	C	C	C	T	C	C	C	T	C

* $p < 0.05$ (Wilcoxon Matched Pairs Test (Zar 1996))

the continuous movement between the two bowls of food was not observed as it was with DDS. Hence, even though almost all of the DDS-laced food was consumed, this consumption took longer than the consumption of food laced with any of the other chemical compounds.

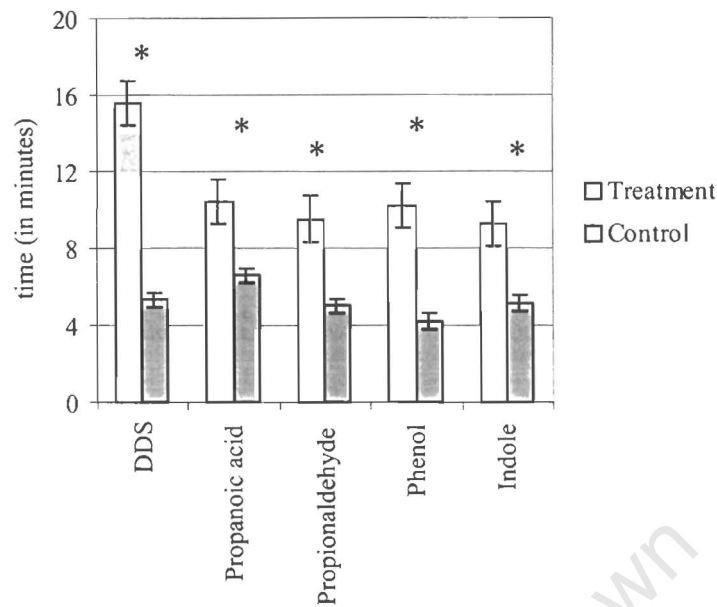


Figure 5.3: Time taken (in minutes) for the cats to begin eating the offered food, treatment and control, until all of the food had been consumed or they no longer displayed interest in the remaining food (Wilcoxon Matched Pairs Test: * = $p < 0.05$).

Reptilian predators:

Similar cafeteria experiments were carried out on Rock Monitors, *Varanus albigularis*. Ten individual Monitors were offered mice that had been variously scented with synthetic Woodhoopoe secretion or sterile saline. Of the ten Monitors that were offered the treated mice, only one Monitor consumed a mouse that had the Woodhoopoe secretion on its fur (Fisher's Exact Test: $p = 0.0011$). Careful note was taken of the actions of the Monitors during these feeding trials. In most cases the Monitors would 'sniff' at the mice with their tongues and promptly turn away from the mice onto which the secretion had been dropped. However, when they encountered mice treated with saline, these were consumed instantly. In the case of the single treated mouse that was

eaten, the Monitor, a pregnant female, approached the mouse and sniffed at it. She picked up and dropped the mouse repeatedly before rolling it in the sand, until it was covered in sand, before eating it. This process took approximately 15 minutes.

Discussion:

Mammalian predators:

Wanless' (1998) experiments conclusively show that cats are sensitive to the odour of proffered food. His experiments showed that cats were able to distinguish between tainted food and food which was in close proximity to a tainted object. In the few cases where cats attempted to eat of the tainted food, the food was soon abandoned and the untainted food was consumed.

Although cats are sensitive to the odour and taste of food, cats will attempt to eat malodorous food that is palatable, even if the consumption of this food takes longer than food that is neither malodorous nor unpalatable. This is supported by the length of time taken by the cats to consume the food that was laced with DDS. Although the food was unpleasantly scented, the scent was not strong enough to completely dissuade the cats from eating the proffered meal. In other cases, such as those of propionaldehyde, phenol and indole the taste of the food alone was sufficient to prevent the cats from completing the meal offered to them. DDS carries an almost over-powering smell of sulphur, whereas the other compounds carry very little or no scent; likewise, the solvents DMSO and water have no apparent scent to the human nose. The solvent DMSO apparently did not have any effect upon the cats as they consumed all of the food that was treated with either it or with water.

A comparison of the quantities of food eaten by the cats during Wanless' experiments and my experiments show that the individual compounds appear to be less distasteful to the cats than the entire secretion. Combining the individual constituents in to the mixture found in the secretion seems to have a synergistic effect in deterring the cats from the food. This synergism may be due to the combination of both odour and taste. Indole is the only compound within the secretion that shows an effect upon food consumption anywhere near as great as the entire secretion.

Thus, I conclude that only the chemicals found within the secretion were causing the aversive reaction in the cats. The fact that the cats readily consumed the food with which they were provided after the completion of the experiment showed that the cats were not avoiding the scented food due to satiation. The cats were still hungry after eating the untainted food, but would rather go hungry than consume food that they found distasteful.

The mass of an average adult Woodhoopoe ranges between 65g (female) and 90g (male), which represents a standard meal for any cat-sized predator. Considering that Woodhoopoes are group-living birds, a group of between two and 12 such meals would be attractive to most predators, until faced with the actual odour and flavour of such a meal. Woodhoopoes being birds that obligately roost communally at night would certainly attract nocturnal predators such as cats and their relatives. Birds which constantly come under predation pressure would benefit from the development of a defence mechanism against predators. The malodorous secretion of *P. purpureus* could have evolved to afford this protection.

Other hypotheses exist to explain the production of this secretion: that it serves to protect the birds against disease and feather degradation, that it serves to deter parasites from the birds and their roost-cavities or that the each family group carries and individual scent and this is being used to mark their territories. It has already been shown that the secretion has anti-microbial properties (see Chapter 3), though it does not seem to deter arthropod parasites (see Chapter 3). The hypothesis of the secretion being a territorial marker has remains untested, however the likelihood of this is slim as, unlike mammals (Burger *et al* 1999), birds have not previously been recorded as marking their territories.

Reptilian predators:

Experiments carried out on the Rock Monitor lizards indicate that they have the same, if not stronger, aversive reaction to the full secretion of the woodhoopoes as cats. Some of the reptiles approached the mice treated with the synthetic secretion before approaching the mice treated with sterile saline. In either case they would flick their tongues over the mice before picking them up. The monitors would not pick up the mice treated with the synthetic secretion, but moved sharply away from them. In contrast to this, they would devour mice treated with saline immediately.

A normal meal for these captive monitors consisted of three to four mice at a time, whereas in this case they were only offered two mice. The fact that the monitors would not even consider eating the mouse treated with the woodhoopoe secretion shows that the secretion was sufficiently distasteful to them that they would rather remain hungry than eat mice treated with the synthetic woodhoopoe secretion. In the only case where

one of the treated mice was eaten, the gravid female monitor scoured the mouse in the dirt in an obvious attempt to remove the source of the malodour from the food.

Most of the reptilian predators that the woodhoopoes in Morgan Bay would come into contact with are diurnal (Branch 1998). This means that these reptiles would probably only be able to prey upon eggs, nestlings and incubating females, as the rest of the family group would not be within the roost chamber during the daylight hours. It is likely that the eggs and nestlings would have a lower concentration of the secretion upon them. The eggs may have a small quantity of the secretion on them from contact with the female, while the nestlings would not have developed the full spectrum of compounds within their own uropygial secretion due to the period of time required for inoculation of the bacterium '*Enterococcus phoeniculicola*' into the uropygial gland (see chapter 3). This would make it most likely that the eggs and nestlings would possibly be the favoured prey of arboreal reptile predators. However, the behaviour of the mother, as well as the secretion that she produces, may be sufficient to protect the eggs and nestlings.

This study shows that the secretion serves as a defence mechanism against both mammals and reptiles, with the bright colouration and odour of the birds possibly serving as warnings to potential predators of their distastefulness.

VI. Conclusion:

Red-billed Woodhoopoes belong to the order Upupiformes, along with other members of the genera *Phoeniculus*, *Rhinopomastus* (Scimitar-bills) and *Upupa* (Hoopoe) (Sibley 1996). The scent associated with Red-billed Woodhoopoes has been noted in Scimitar-billed (Jackson 1938, Steyn 1999) and Violet Woodhoopoes (Du Plessis pers. comm.).

Eurasian (*Upupa epops*) and African hoopoes (*U. africana*) nestlings have an enlarged uropygial gland that secretes a reddish-brown oil which has a very strong odour (Heinroth 1944, Sutter 1946, Skead 1950, Vaurie 1973). Combined with this, they also squirt liquid faeces at potential predators. Once the nestlings are ready to leave the nest, the gland has returned to a normal size and no longer has any detectable odour (Sutter 1946). The only anti-predator mechanisms employed by the adults is the ejection of liquid faeces by the incubating female; no such behaviour by male hoopoes has been recorded (Sutter 1946, Skead 1950, Vaurie 1973).

The secretion produced by *P. purpureus* consists of seven compounds, the bulk of which is cholesterol. This follows the norm for most birds, whose uropygial secretions are comprised mostly of fats and waxes (Jacob 1982, Levy and Strain 1982, Jacob and Hoerschelmann 1985, Livezy *et al* 1986). This does not, however, account for the strong odour (Steyn 1999) and colouration of the secretion.

Some birds and mammals sequester chemicals directly from their diet (Cott 1946, Ziegler 1971, Dumbacher and Pruett-Jones 1996). Similarly, animals are able to sequester compounds that are produced by bacterial fermentation within the gut of the animal (Cott 1946, Ziegler 1971, Dumbacher and Pruett-Jones 1996). The results of tests done upon the secretion when the woodhoopoes were fed an artificial diet, as

opposed to a natural diet, suggest that, except for the possibility of dimethyl disulfide and isovaleraldehyde, the chemicals within the secretion are not a product of their diet.

Bacteria readily produce aromatic and other compounds through the metabolism of their substrate (Allison 1978, Camargo *et al* 1982, Padilha *et al* 1995, Cabral *et al* 1997, Krings and Berger 1998, Arp 1999, Khan *et al* 1999, Taniguchi *et al* 1998, Rehman 1999, Birtkett *et al* 2000, Cass *et al* 2000, Higaki *et al* 2000, Rose *et al* 2000). The secretion that is produced by the bird is modified by the metabolic action of a single species of symbiotic bacteria that is resident within the uropygial gland. This bacterium has not previously been identified and for this reason is proposed as the type species '*Enterococcus phoeniculicola*'.

Bacterial symbionts of birds do not only present themselves as apparently harmless commensals (Cooper 1990, Cooper 1993, Hubálek *et al* 1995, Singleton and Given Harper 1998, Burt 1999). Many bacteria found within nests and on the birds themselves are part of their normal flora. Others may be opportunistic or obligate pathogens. Bacteria and fungi need not be lethal, but can cause damage to feathers. This could cause the death of the bird at a later stage.

Birds use their uropygial oils for water-proofing and feather maintenance, spreading the oils over the feathers with the tip of the bill. *In vitro* testing of bacteria against a quantity of the artificial secretion inhibited bacterial growth. Hence daily preening could serve to disinfect the feathers of any bacteria that are not constituents of the birds' normal flora. This would, in effect, protect the birds from feather degradation and pathogens. Birds ingest some of their uropygial secretion when preening (Elder 1954).

The secretion could therefore also afford the birds protection against pathogenic gut bacteria.

Preening the feathers not only water-proofs them and cleans them, but may also remove arthropod ectoparasites (Clayton 1991). Many types of arthropods are found on birds: mites, lice, ticks, fleas and flies. Many of the arthropod symbionts are not permanent residents on birds, residing rather within the nest material and only coming into contact with birds when feeding. The synthetic secretion of the Red-billed Woodhoopoe was tested only upon feather mites found upon the primaries of the birds and did not have any apparent effect upon these mites. Feather mites are relatively host-specific (Clayton 1991) and it is therefore conceivable that they have evolved a concomitant resistance to the effects of the secretion. Alternatively, feather mites may not cause sufficient damage to feathers as to select for the development of mite-specific defences.

Birds not only have to deal with microscopic threats but also with macroscopic ones. Mammals and reptiles are known predators of woodhoopoes (Ligon and Ligon 1978). Wanless' (1998) experiments showed that cats are extremely sensitive to quantities of the synthetic secretion being placed on otherwise highly palatable food. Cats rather remain hungry than consume food that they find unpalatable. This was confirmed by the fact that the cats consumed untainted food that was later provided. My results show that three of the six major groups of constituent compounds (indole, phenol and propionaldehyde) are responsible for this reaction by the cats. The aversive reaction is not as marked when the food is treated with only a single compound as it is when treated with the full secretion. Having the compounds together in one 'mix' appears to have a synergistic aversion effect upon the cats.

The secretion has a marked effect upon Rock Monitor Lizards. Monitors are diurnal predators and would therefore only be exposed to birds carrying the secretion if they had to approach a nest containing a female with eggs or nestlings. Cats are nocturnal predators and are therefore more likely to come upon roost cavities containing entire families of woodhoopoes. For this reason, cats may have become slightly desensitised to the secretion. Woodhoopoes are also depredated by *Accipiter* raptor species. Birds are not generally known to have a well developed sense of smell (Burton 1976, Mason and Clark 1998, Rangen *et al* 2000). Raptors hunt by means of aerial attack, without the chance to smell their prey as mammals and reptiles do. It is therefore unlikely that this secretion affords the woodhoopoes any protection against raptors.

The use of chemical defence by birds against predators is relatively well noted. In most cases the chemicals are sequestered, directly or indirectly, from their diet. Less well documented is the use of chemicals by birds against pathogens and parasites and in cases where this has been observed it has been through the topical application of other organisms that produce these chemicals. Apparently birds of the family Upupidae are the first recorded to produce these chemicals from their uropygial gland. Hoopoes produce anti-predator chemicals only as nestlings while Red-billed Woodhoopoes produce chemicals against pathogens, parasites and predators from an early age through adulthood. This appears to be the first description of this type of chemical defence; as well as being the first description of this type of symbiotic relationship.

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Appendix A: Using Others to Protect Oneself Chemically.

Summary:

Monteiro's Hornbills, *Tockus monteiri*, are endemic to northern Namibia and southern Angola and make use of millipedes, *Harpagophora diplocrada*, also endemic to this region, in their nest material and nest plugs. The millipedes produce a yellow, odiferous secretion that is likely to contain quinones or hydrogen cyanide, both of which have been identified from the defensive secretions of other millipede species and inhibit microbial growth. I found both bacterial and fungal numbers to be significantly higher in unoccupied nest-boxes than in occupied nest-boxes, even though conditions within occupied nest boxes would be more conducive to microbial growth. I think that this is due to the presence of the millipede secretion within the nest material of occupied boxes. I placed *T. monteiri* nestlings in groups of four within nest-boxes and allowed them to acclimate for three days to the microbial population within those boxes. After a period of time I treated them with a dilute form of the secretion and then compared samples taken after this treatment with samples taken from birds treated with sterile saline. I found that birds treated with the dilute millipede secretion had significantly lower bacterial numbers than control birds. From both of these results I deduce that the birds make use of the millipedes as fumigants for their nest sites.

Introduction:

All Hornbill species nest in cavities (Kemp 1995). In all of these, excluding the two species of the genus *Bucorvus* (Kemp and Begg 1995), the female seals herself into the nesting cavity for the duration of the breeding period. In Monteiro's Hornbill, *Tockus monteiri*, a species endemic to the arid woodlands of northern Namibia and southern Angola, the female seals herself into the nest cavity for a period exceeding 50 days for the purpose of laying and incubating eggs and attending to the young nestlings (Kemp

1973). While in the nest cavity, the female undergoes a complete moult that renders her flightless and vulnerable (Kemp 1973).

The cement of the nest cavity seals consist of a mixture of mud, faeces and food items (Kemp 1973, Poonswad *et al* 1983, Kemp 1995), which include arthropods and fruit, depending upon the Hornbill species in question (Poonswad *et al* 1983, Kemp 1995). Most Asian Hornbill species apparently make use of fruit within their nest plug cement, while arthropods are more often used by the African species. Of the African species that make use of arthropods in their nest plugs, four have been positively identified as making use of crushed millipedes (Kemp 1995). These four species are distributed through the entire habitat spectrum of Africa, from the most arid areas of Namibia to mangroves, montane and coastal evergreen forests (Kemp 1995). In the case of *T. monteiri*, millipedes are selected, crushed up and placed in the mud and faecal mixture used to seal the cavity (Kemp 1995) as well as into the material used to line the nest.

Many species of millipedes secrete substances from repugnatorial glands present along the lateral sides of the body (Lawrence 1981). The secretion is composed primarily of benzoquinones and hydrogen cyanide, which serves as protection against predators (Eisner *et al* 1965, Krebs 1964, Lawrence 1972, Attygalle *et al* 1993, Williams *et al* 1997). The secretion also serves to protect the millipedes against parasites and pathogens (van der Walt and McClain 1990, Williams *et al* 1997).

Birds play host to many species of bacteria, some of which are the normal flora of the birds, while others may be opportunistic pathogens or obligate pathogens (Cooper *et al* 1986, Cooper 1990, Cornelissen *et al* 1991, Cooper 1993, Hubálek *et al* 1994). Many species of birds re-use nests for more than one breeding season without removing the

previous season's nesting material (Singleton and Given Harper 1969) and, hence, lowering the microbial population of the nest-site. Bacterial and fungal species not only pose a threat to both nestling and adult health, but also to their plumages (Hubálek 1976, Burt and Ichida 1999). This may be a significant problem to birds that roost in cavities and especially to female Monteiro's Hornbills that undergo a complete moult during the nesting period (Kemp 1995). Feather degrading bacteria and fungi would damage not only the newly grown feathers of the female, but also those of the nestlings.

The question now arises: can, and do, birds protect themselves against bacterial onslaught? It appears that some species of birds and mammals have developed a variety of methods of lowering their bacterial loads. First, several hundred species of passerines display the behaviour known as anting, during which birds select only ants that secrete acids or other aromatic compounds. During this process the ants are squeezed and then vigorously rubbed over the plumage (Judson and Bennett 1992, Clayton and Vernon 1993, Clayton and Wolfe 1993). Secondly, birds such as European Starlings, *Sturnus vulgaris*, use aromatic species of plants for placement in their nest material (Clark and Mason 1985, Mason and Clark 1986, Clark and Mason 1988). The selected vegetation apparently inhibits certain larval stages in arthropod ectoparasite growth and inhibits bacterial growth. Thirdly, Redbilled Woodhoopoes, *Phoeniculus purpureus*, play host to symbiotic bacteria ('*Enterococcus phoeniculicola*') that reside in their uropygial gland. This bacterial species modifies the preen oil produced by the birds. I have shown, by means of *in vitro* testing, that this modified oil inhibits bacterial growth (see Chapter 4).

Although some Hornbill species bring millipedes into their nests and incorporate them into the plugs used to seal the female into the nest cavity, it is not known why this is done. With the knowledge that some millipedes secrete antimicrobial substances, I aim to determine whether the behaviour of crushing millipedes into the nest plug cement, and their nest material, serves to reduce the number of bacteria and fungi found within the nests, and upon the Hornbills themselves.

Methods:

I took samples of nest material from nest boxes erected in the Daan Viljoen Nature Reserve outside Windhoek, Namibia (22°30'S; 16°58'E). I collected nest material samples from ten individual boxes in which there were no resident birds (unoccupied) as well as from 13 individual boxes in which Monteiro's Hornbills, *Tockus monteiri*, were breeding (occupied). The occupied nest boxes were sealed with a plug consisting of faeces, mud and crushed millipedes. The nest material samples from both occupied and unoccupied nest boxes were examined and fragments of millipede exoskeleton were found in both.

I placed one gram of nest material in 10ml of water and then serially diluted this solution down to 10^{-8} ; spread-plating 100 μ l of each of the series of dilutions in duplicate onto nutrient agar (NA) plates and incubating them overnight at 37°C. I also spread-plated, in duplicate, dilutions of 10^0 to 10^{-3} of the solution onto potato dextrose agar (PDA) plates and also incubated these overnight at 37°C. After the incubation period, I carefully counted the number of colonies growing upon both the NA plates and the PDA. I used two different types of media due to the fact that NA promotes bacterial growth, while PDA promotes fungal growth. I analysed the results obtained from the

culturing of the microbes found in the nest material using the T-test assuming unequal variances.

I collected *Harpagophora diplocrada* (Attems 1909), a species of millipede that is near-endemic to Namibia, in the Daan Viljoen Nature Reserve during their most active period, immediately after rain. This species of millipede is used by *T. monteiri* in their nest material and nest plugs. After I had collected the millipedes, I gently agitated the individuals until I noted the secretion forming in tiny droplets along the lateral sides of the millipede. The latter secretion has a very characteristic scent. I collected the secretion using small pieces of sterile filter paper, which I rubbed along the line of glands on the sides of the millipedes using sterile forceps. I placed these pieces of filter paper in just enough sterile water to remove the secretion from the paper.

I collected Hornbill nestlings from various nest sites and placed them in nest boxes in groups of four in order to control for age and group size. After the groups were established, the nestlings were left untouched for three days to allow the nestlings to acclimate to their new living conditions – the change in competitive stresses and microbial flora of their new living environment. The Kruskal-Wallis single-factor analysis of variance test was used to determine that these were independent samples ($H_{0.05,4,4,4} = 9.846$, $p = 0.007$) and were therefore treated as such.

I swabbed an area of approximately 3cm^2 between the scapulae of each individual nestling with a sterile cotton swab dipped into sterile water. I then re-swabbed the same area with the extraction of the millipede secretion. I re-swabbed the same area of skin between the scapulae of the individual nestlings with sterile water the following day and finally two days following that. I serially diluted the swab samples down to 10^{-3} and

spread-plated 100µl of these dilutions, in duplicate, onto NA plates and PDA plates, which I then incubated at 37°C overnight. Another 12 nestlings, placed in a further three nest boxes were swabbed only with sterile water. The number of bacterial and fungal colonies that had grown on the plates overnight were noted and the results for the treated birds were compared with those of the control birds using ANOVA and the Tukey test as a *post hoc* test.

Results:

Microbial loads: occupied versus unoccupied nest-boxes:

There were significantly higher bacterial numbers in unoccupied than occupied nest-boxes (T-test Assuming Unequal Variances: $t_{crit} = 2.120$, $p = 0.048$). Fungal numbers

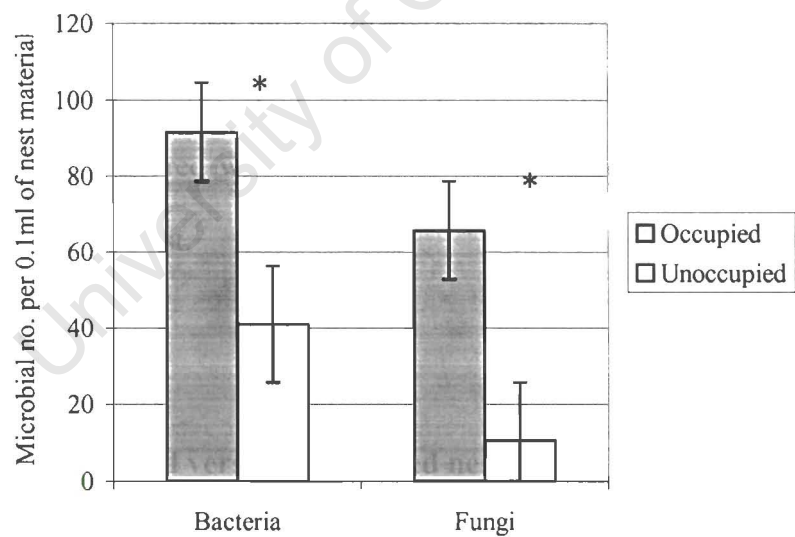


Figure A1: A comparison of bacterial and fungal numbers found in occupied *Tockus monteiri* nest boxes ($n = 13$) and presently unoccupied ($n = 10$) nest-boxes. A significant difference (*) was noted for both bacterial ($p = 0.048$) and fungal ($p = 0.028$) concentrations (T-test Assuming Unequal Variances).

were also significantly higher in unoccupied nest-boxes than occupied nest-boxes (T-test Assuming Unequal Variances: $t_{crit} = 2.160$, $p = 0.028$) (see Figure A1 and A2).

Microbial loads: nestlings treated with millipede secretion versus untreated nestlings:

The ANOVA analysis showed a significant difference in bacterial numbers between nestlings treated with the millipede secretion and those treated with sterile saline ($F_{2, 30} = 23.02$, $p < 0.0001$). The Tukey test was used as a *post hoc* test to verify this result. I

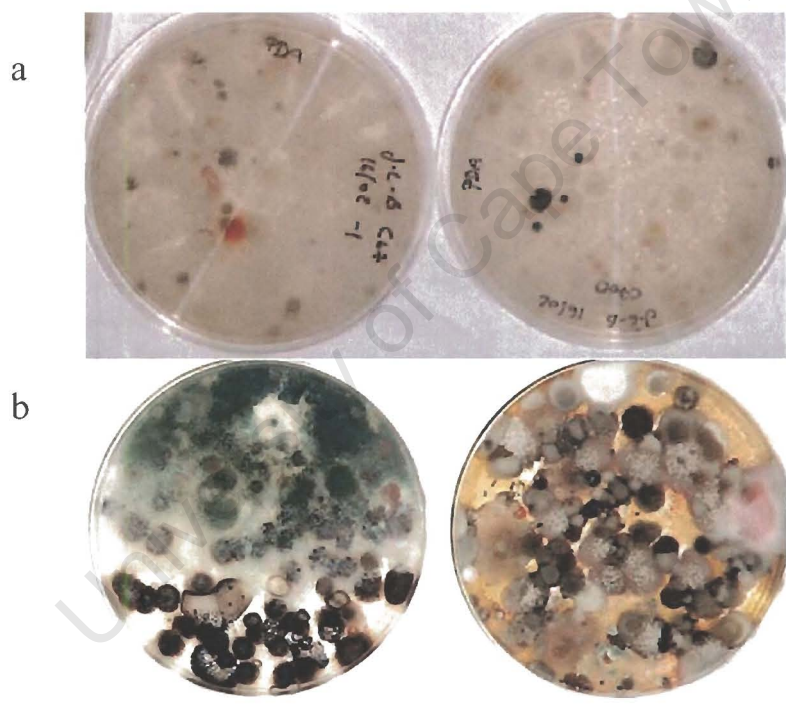


Figure A2: The difference in fungal concentrations found in occupied (a) versus unoccupied nests (b).

found significantly higher bacterial numbers on untreated nestlings as opposed to treated nestlings on all days that samples were taken: day 0 vs. day 1, $p = 0.031$; day 0 vs. day 3, $p < 0.0001$; day 1 vs. day 3, $p = 0.0097$ (Figure A3.). I never found any

fungal spores on the nestlings, before or after the treatment, on the treated or control birds. Figure A3 clearly shows the continued decrease in bacterial numbers found between the scapulae of Monteiro's Hornbill nestlings, even three days after the application of the millipede secretion

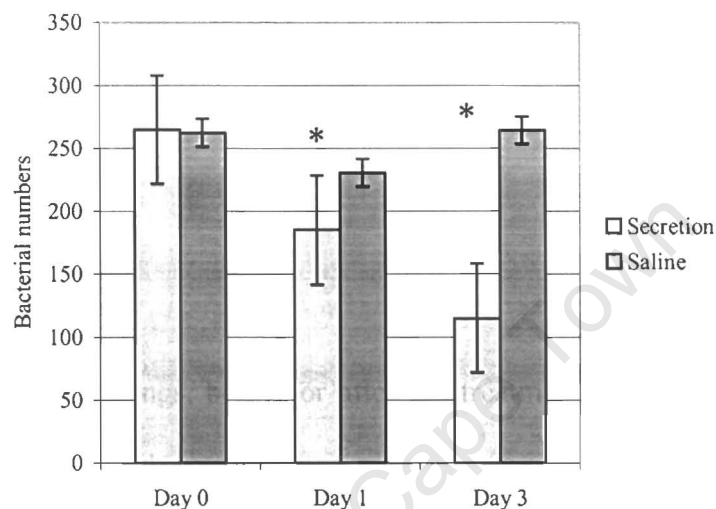


Figure A3: Trends in bacterial numbers found on the 3cm² area of naked skin between the scapulae of *T. monteiri* nestlings (n = 12; ANOVA: $F_{2,30} = 23.02$, $p = 0.00097$).

* denotes a significant difference in bacterial numbers ($p < 0.05$) on treated versus untreated nestlings.

Discussion:

Microbial loads: occupied versus unoccupied nest-boxes:

An enclosed nest cavity is the ideal environment for microbial population explosion: it is warm, humid, has plenty of decaying matter, such as faeces, feathers and food-stuffs, and contains young nestlings with undeveloped immune systems. Hornbills also do not

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remove old nest material from nests. Considering all of these factors, the relatively small microbial populations found within *T. monteiri* nest-boxes might be considered quite surprising. However, if one considers the secretion produced by millipedes, some of which are known to produce microbial toxins and inhibitors (Shpall and Frieden 1991, Williams *et al* 1997), the microbial concentrations found within occupied *T. monteiri* nest-boxes is not that surprising. Monteiro's Hornbills catch and crush up millipedes and place them in their nest material some days before the female enters the nest-box and seals herself in. The act of picking up the millipede will induce it to release its defensive secretion before it is crushed; exposure of nest material to this secretion would lower the microbial concentration found within the nest material before the female takes up residence within the box.

It is likely that the secretion produced by *H. diplocrada* contains quinones. It has a distinctive odour and causes discolouration of the skin, similar to that of the defensive secretion produced by *Tylobolus* sp. and quinones (Shpall and Frieden 1991). Quinones are often found in millipede secretions that are antimicrobial (Williams *et al* 1997).

Microbial loads: nestlings treated with millipede secretion versus untreated nestlings:

The initial decrease, between day 0 and day 1, in bacterial numbers, found on the skin between the scapulae of the chicks, could be attributed to the observer removing the bacteria due to swabbing. However, this would not explain the significant difference in bacterial numbers between the treatment and control birds and the continued and significant decrease in bacterial numbers up to and including day 3.

Although Hornbills do not actually apply the millipede secretion to themselves, I believe that the dilute secretion applied to the nestlings would mimic the action found in the nests as the secretion volatilises from the nest material. This may also explain the lack of fungi found on the nestlings in both the control and treatment boxes, considering the lowered number of fungi within occupied nest boxes, as opposed to unoccupied nest boxes. It has been shown that proximity to an antimicrobial agent can lower microbial numbers on the proximal object / animal (Clayton and Wolfe 1993).

My study shows that the dilute form of the secretion significantly lowered the bacterial numbers on the nestlings, even days after the application of the secretion. Van der Walt and McClain (1990) showed a similar pattern when carrying out *in vitro* testing of the secretion produced by a species of millipede found in the Kalahari. *In vitro* testing of this secretion against bacteria showed that inhibition of bacteria was greatest 96 hours after the stimulation of secretion production.

Although the hypothesis that some Hornbill species use millipedes in the nest-plug cement for added moisture and binding, may be a plausible one, it does not explain the use of millipedes within the nest material of *T. monteiri*. The results of my study show that the secretion produced by the millipedes does have antimicrobial and antifungal properties and this may provide one explanation for the behaviour of the Hornbills placing the millipedes into their plugs and nest material. Some birds make use of aromatic plants to lower parasite and microbial numbers within their nest material (Clark and Mason 1985, Mason and Clark 1986, Clark and Mason 1988). In a semi-arid environment such as Namibia, where rainfall and vegetation is relatively sparse, it may not always be feasible to make use of plants to 'fumigate' one's nest. Hence, it may be

more feasible for cavity nesting species, such as *T. monteiri* to make use of arthropods which secrete volatile chemicals, such as millipedes.

The use of millipedes within the nest plug does not seem to deter predators in any way as a number of nest boxes were predated by Rock Monitor Lizards, *Varanus* sp., and by baboons, *Papio cynocephalus ursinus*, even though there was an abundance of food due to the high rainfall.

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