The Anatomy and Histomorphology of the Uropygial Gland in New Zealand Endemic Species

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Abstract

Considering that there are more than 10,000 species of birds on earth, and that the uropygial gland is the most prominent integument gland in this vertebrate group, it is puzzling that little is known about its morphology and function. The current hypotheses for the function of the uropygial gland can be placed into four groups: 1) feather maintenance; 2) water-proofing; 3) intraspecific communication/health; and 4) defence against predators and/or parasites. Several studies have examined these hypotheses, although no general function for the uropygial gland has been established.

This thesis aimed at reducing the gap in knowledge of the uropygial gland by investigating New Zealand birds. The purpose of this study was to examine the anatomical and histological structure of the uropygial gland in New Zealand birds and to investigate the defence hypothesis as a function of the gland specifically in brown kiwi (*Apteryx mantelli*).

Anatomical and histological analyses of the uropygial glands from brown kiwi, great spotted kiwi (*Apteryx haastii*), hihi (*Notiomystis cincta*), New Zealand bellbirds (*Anthornis melanura*), tui (*Prosthemadera novaeseelandiae*), and saddleback (*Philesturnus carunculatus*) were carried out. The anatomy and histology of all glands were compared both within family and order and to those available from other species worldwide. The defence hypothesis function of the uropygial gland was investigated using the tick species *Ixodes anatis* from the skin of brown kiwi.

This study revealed a range of uropygial gland characteristics in the kiwi, hihi, New Zealand bellbird, tui, and saddleback that were not know to previously exist in other species. For example kiwi uropygial glands were found to possess eight primary sinuses. Comparison of the New Zealand passerines revealed that bellbirds possess the largest gland in relation to body size out of the four species. The uropygial secretion of brown kiwi may play a role

in parasite repellence as both males and female ticks were deterred from the secretion. Based on histomorphology I suggest that rather than a single function, the gland may have species/group functions. However, this hypothesis still remains enigmatic due to the lack of birds studied to date.

Acknowledgments

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Chapter 1: Introduction.

The evolution of flight in the avian fauna has elicited many adaptations not seen in other vertebrate groups. These include the loss of internal organs (for a detailed review see Campbell & Reece, 2005) and vast changes in the integument (Kardong, 2012). The production of the keratinised feather is the most obvious integumentary adaptation for a bird's aerial mode of life. However, the integument itself has also adapted to become a loose elastic organ which permits efficient movement during flight (Stettenheim, 2000). Even though the integument is thin, it is still composed of an outer epidermis and an inner dermis (King & McLelland, 1985). The epidermis is keratinised and is comprised of keratinocytes – keratin producing cells – while the dermis supplies growing feathers with essential nutrients, and allows for voluntary feather movement (Kisia, 2010).

The integument as a whole is a highly lipogenic organ (Lucus and Stettenheim, 1972). During keratinisation of the epidermis, phospholipids are bound within the superficial areas. The overall amount and type of phospholipid present depends on the species of bird and the location on the body; however, they are always derived from lamellar bodies (Menon & Menon, 2000). These phospholipids serve a thermoregulatory purpose and are known to assist in evaporative cooling of the bird along with other variable functions (Menon & Menon, 2000). Some epidermal phospholipids can be highly specialised; for example in the Japanese Crested Ibis (*Nipponia nippon*) black phospholipids from the head region formed during the mating season are utilised in 'cosmetic colouration' (Uchida, 1970). These phospholipids are smeared over the body with the bird boasting a 'change in colour' without a moult ever taking place (Uchida, 1970).

In comparison to other vertebrate groups, birds do not possess a wide variety of specialised integumentary glands. This is in direct contrast to the large number of integumentary glands observed in mammals (e.g. eccrine, apocrine

and sebaceous glands). The uropygial gland (UG) is an integumentary gland specific to this group – yet its exact function remains highly speculative. It is thought that the UG is analogous to the mammalian sebaceous gland which functions in oil production (King & McLelland, 1985; Salibian, & Montalti, 2009). In mammals, sebaceous glands have a holocrine structure and small, individual glands cover the entire integument (except hands, feet, hooves, or paws) (Marieb & Hoehn, 2007). The secretion is most commonly deposited into hair follicles which aids in maintaining the condition of the hair (Kardong, 2012). The secretion also functions to reduce water loss from the body, and it possesses bactericidal properties in some species (Marieb & Hoehn, 2007).

1.1. Uropygial gland overview

Birds are exposed to terrestrial pressures similar to mammals and reptiles yet the structure of their integument differs markedly from these animal groups (Menon, & Menon, 2000). One of the major differences is the development of the UG which like mammalian sebaceous glands produces oils (King & McLelland, 1985; Sara *et al.* 2006).

In extant birds, the UG has a shared basic structure, although many specific differences have been noted (pages 204-213 in Jacob & Ziswiler (1982); Sara *et al*. 2006; Martín-Vivaldi *et al.* 2009). In all species of bird examined to date, this gland is present throughout embryonic development, however, it is not necessarily present in the adult (Montalti, & Salibian, 2000; Salibian, & Montalti, 2009; Martín-Vivaldi *et al*. 2009). This gland may be considered a primitive feature of the group aves, because orders which do not possess the gland as adults are possibly exhibiting a form of secondary specialisation (Montalti, & Salibian, 2000). Since the function of this gland is not yet known, research into its existence in different species is imperative to the discovery of the evolutionary drive causing its development. Considering that there are about 10,000 bird species wordwide, it is very interesting that only 16% of birds UG's have been investigated anatomically. With such a small number the information to be gained from

future investigations is likely to be substanstial and will contribute to the overall understanding of both the structure and function of this gland.

1.2 Uropygial gland morphology

From investigations of the UG in birds to date, the UG is known to be situated dorsally and medially in the synsacrocaudal region of the bird's body and, when present, is always visible to the naked eye (Figure 1.1; Jacob, & Ziswiler, 1982; Martín-Vivaldi *et al*. 2009; Harem *et al.* 2010). It is most commonly a bilobed organ, which varies in both size and shape, depending on the species (Stettenheim, 2000; Salibian, & Montalti, 2009). Each lobe contains both the secretory tissue which produces the oil, and an intricate duct system which relays the secretion to the papilla, the route to the skin's surface (Figure 1.2; Jacob, & Ziswiler, 1982; King & McLelland, 1985; Salibian, & Montalti, 2009). The papilla is located just above the tail (King & McLelland, 1985; Stettenheim, 2000) and has a characteristic nipple-like appearance (Jacob, & Ziswiler, 1982). Most birds also have a tuft of downy feathers (Jacob, & Ziswiler, 1982; Stettenheim, 2000) arranged around the papilla, which aids in anointing the bill with the oily secretion (Lucas & Stettenheim, 1972; King & McLelland, 1985; Stettenheim, 2000). Once on the bill the secretion is spread through the plumage.

Figure 1.1: Photo showing the location of the UG in a penguin (arrow). The gland is located in the same general place in all bird species studied thus far. Photo sourced from www.google.co.nz/images.

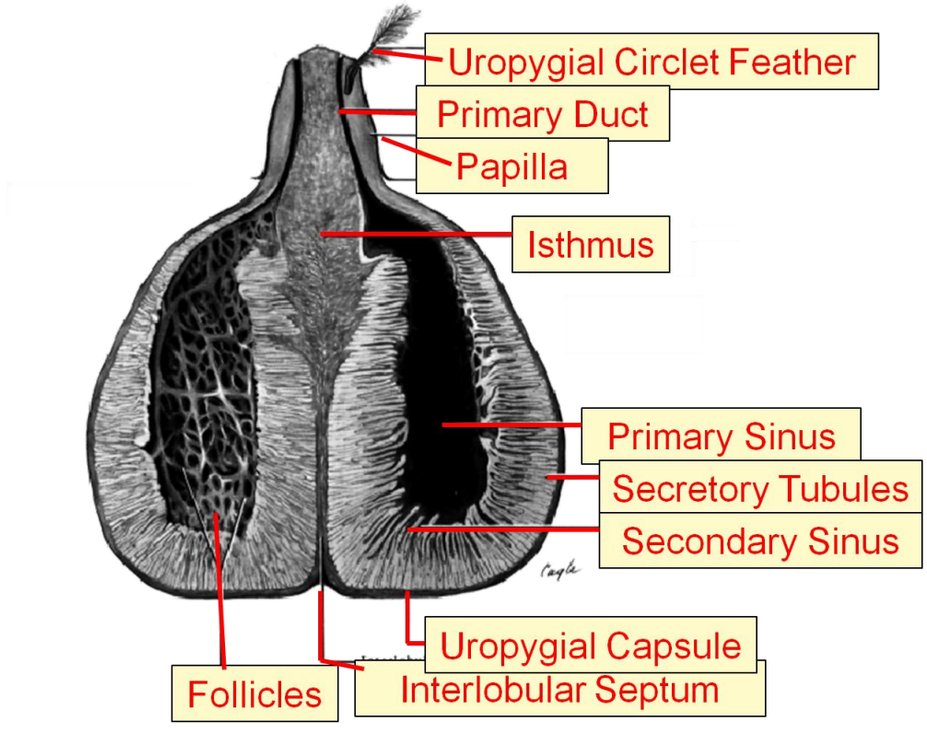

Figure 1.2: Modified drawing of a UG illustrating anatomical organization. (Jacob & Ziswiler, 1982).

The papilla is clearly separated from the lobes of the gland by an isthmus formed by very strong connective tissue (Figure 1.2; Lucas & Stettenheim, 1972). Jacob and Ziswiler (1982) demonstrated the morphological differences between glands in a variety of birds. They found that the largest UG when expressed as a percentage of body weight occurred in the Little Grebe (*Tachybaptus ruficollis*) – it accounted for 0.61% of total body weight. The smallest gland was found to occur in fruit pigeons (genera: *Ducula* and *Ptilinopus*), where it represented <0.02% of total body weight. Comparative studies of this sort are important in discovering the selective pressures driving the UG development and thus differences between differing groups of birds (Salibian, & Montalti, 2009).

The appearance of the lobes differs in many species and may have taxonomic significance (Figure 1.3) (Elder, 1954; Jacob & Ziswiler, 1982). The lobes are most commonly joined for approximately two-thirds of their length. An example of this is seen within the Strigiformes (owls, *Tyto alba;* Figure 1.3) where it appears the UG only consists of one lobe. It does however consist of two lobes which are joined for their entire length causing the gland to appear homogenous (Jacob & Ziswiler, 1982). As Figure 1.3 illustrates, the shape of the UG can range from long, short, wide, skinny, round to flat.

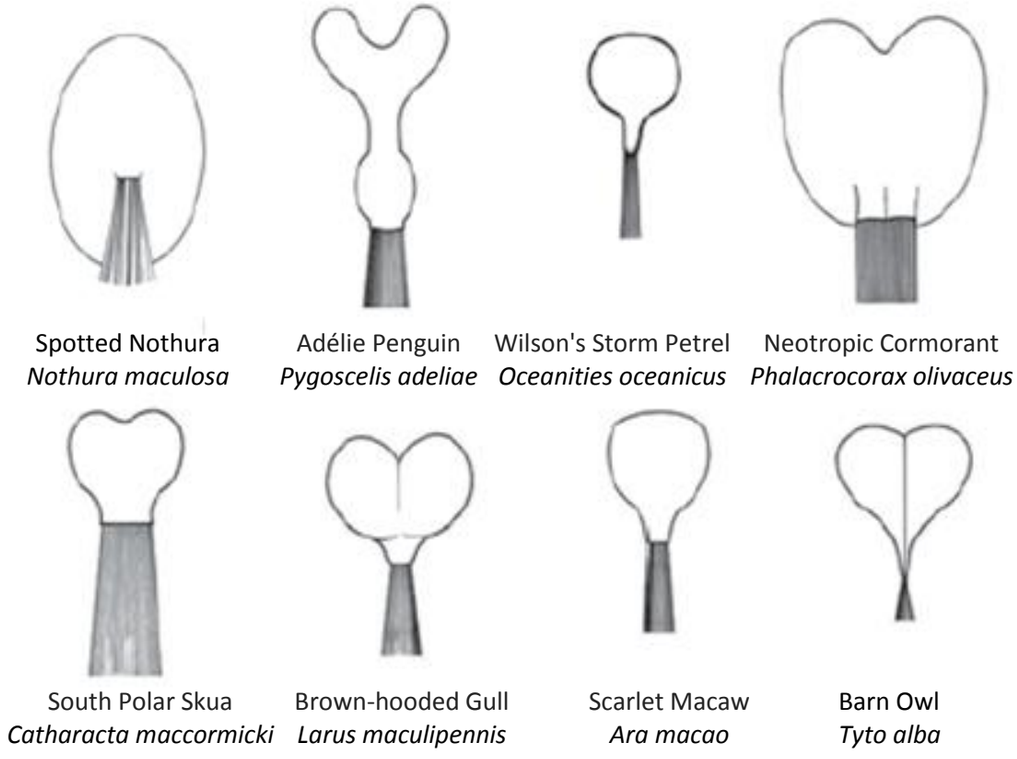

Figure 1.3: Morphological diversity of UG lobe shape and size (Salibian & Montalti, 2009).

The papilla is another structure which differs greatly in shape and size between species; including duct characteristics (for detailed review see table 1 (page 204) in Jacob & Ziswiler, 1982). In most birds the papilla contains two ducts although this differs between species. For example Nitzsch (1840) found that the Hoopoe (*Upupa epops*) has one broad opening characterised by a single duct in the papilla while species of

Procellariiformes and Pelecaniformes have more than two (Jacob & Ziswiler, 1982). Jacob and Ziswiler (1982) describe three duct types: compact, delicate, and the 'wart-like papilla of the Passeriformes'. Compact ducts are characteristic of papillae where the connective tissue of the interlobular septum surrounds each duct and causes them to be narrow. Delicate ducts on the other hand are wide ducts which almost fill the entire space within the papilla region. They lack the compact connective tissue and most commonly the wide spaces are produced by the continuation of the primary sinuses towards the apex of the papilla (Jacob & Ziswiler, 1982; Lucas & Stettenheim, 1972). The papilla in the 34 species of passerine described to date is distinct in that it holds a specialised valve. It is a type of delicate duct system which holds two connective tissue lamellae. These valve-like structures are thought to prevent the backflow of UG secretion from the papilla back into the lobes.

Each papilla is characterised by the presence of two orifices (duct outlets) which usually appear as slits (again this is species dependent and more orifices or less orifices may exist) (Jacob & Ziswiler, 1982). These openings allow the secretion to be expelled onto the uropygial circlet feathers. The uropygial circlet then acts as a brush, efficiently guiding the secretion onto the bill for preening (Elder, 1954). The structure of the uropygial circlet feather is a combination of downy feather and semi-plume (Johnston, 1988). Johnston (1988) described three uropygial circlet feather types: type 1, a modified down, and types 2 and 2a modified semiplumes (Figure 1.4). Johnston (1988) found that type 1 was the most commonly occurring feather type. Jacob and Ziswiler (1982) postulated that these feathers were once a part of the dorsal-caudal tract and as the papilla evolved they became separated forming the circlet around the papilla. Jacob and Ziswiler (1982) describe three main arrangements of the circlet feathers around the papilla: 1) the feathers circle all orifices forming a single tuft, 2) the feathers circle each individual orifice forming multiple tufts, and 3) a row of feathers is apparent between each orifice (Figure 1.5). Whatever the case, there can be great deviation from these arrangements and many transitions between them exist – thus their taxonomic importance is debatable and will only be determined as more species are examined in each avian order. The papilla itself is devoid of feathers, a-part from those in the uropygial circlet (Lucas & Stettenheim, 1972).

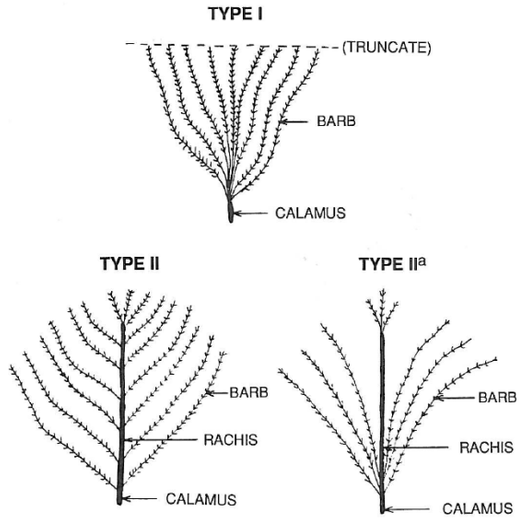

Figure 1.4: Diagram of uropygial circlet feathers described by Johnston (1988). Type 1 = modified down feather; type 2 = modified semiplume; type 2a = modified semiplume.

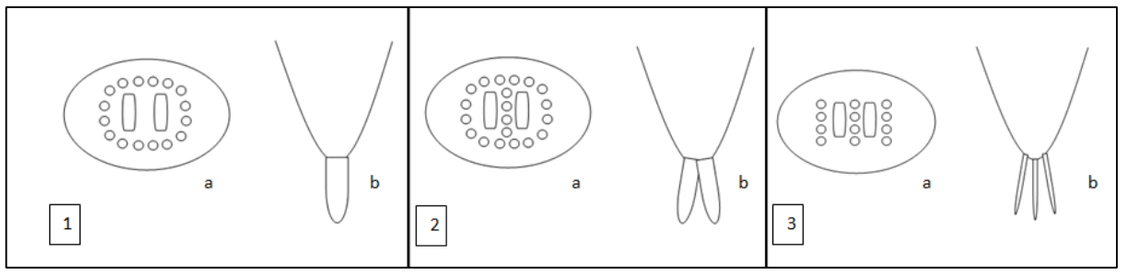

Figure 1.5: Illustration showing the three different uropygial circlet arrangements (a = dorsal view of papilla showing feathers (hollow circles) surrounding orifices (slits); b = longitudinal view showing tuft arrangement). 1 = single tuft (arrangement 1); 2 = double tuft (arrangement 2); 3 = individual rows (arrangement 3). Illustration by author.

1.3 Uropygial gland histology

Because the UG has many proposed functions it is surprising to find a lack of information with regards to its histological organisation (Salibian, & Montalti, 2009; Harem *et al*. 2010). In fact, only 0.2% of bird species UGs have been studied histologically – a surprisingly small number considering there are over 10,000 known species of birds. The few studies carried out show that the UG's histological structure corresponds to that of the mammalian

sebaceous gland (Elder, 1954; Bhattacharyya, 1972). Sebaceous glands of mammals range from simple to complex structures depending on the components required in the secretion. Studies to date have shown that the UG has a holocrine arrangement similar to the sebaceous gland, thus secretion can be stored in ducts and expelled through the papilla when required.

Mammalian sebaceous glands are known to consist of differentiating, volumating cells which degenerate and rupture towards the lumen producing secretion (Kanitakis, 2002). The ducts of sebaceous glands open into hair follicles where the secretion aids in nourishing the hair and cells of the skin (Kardong, 2012). There is a resident microbiota on the skin, which often penetrates into the pores of the sebaceous glands. Bacteria present are most commonly commensal or symbiotic and they restrict the entry of pathogenic micro-organisms across the skins surface (Grice *et al.* 2008). Due to the similarity in histology between the UG and the sebaceous gland, UG secretions may have a role in maintaining the microflora of the avian integument in much the same way as sebaceous glands of mammals. However, to date the microbiology of the avian integument and its relation to the UG has not been investigated.

1.3.1 Uropygial gland capsule

In the 0.2% of birds studied histologically, the UG is bounded by dense connective tissue known as the capsule (Bhattacharyya, 1972; Lucas & Stettenheim, 1972; Jacob & Ziswiler, 1982). Jacob and Ziswiler (1982) and Lucas and Stettenheim (1972) have determined the main components of the capsule to be elastic, collagenous fibres, which are arranged in a dense network around the gland. The capsule of some species (e.g. members of Coraciiformes and Psittaciformes) may contain melanine granula on the dorsal side of the gland, which produces pigmentation and causes the appearance to become spotty or very dark (Jacob & Ziswiler, 1982). Blood vessels, lymph vessels, and nerve fibres are intertwined within the capsule walls, which again is similar to the structure of mammalian sebaceous glands (Elder, 1954; Jacob & Ziswiler, 1982). The studies performed to date have found this capsule to

be devoid of the characteristic bands of smooth muscle tissue present around the glands of mammals; however, the odd solitary smooth muscle fibre may be present in the UG capsule of some bird species (Jacob & Ziswiler, 1982).

The capsule extends ventrally down between the lobes to form the interlobular septum. Jacob and Ziswiler (1982) describe it as only a thin layer although when reaching the papilla it can become very thick. Like the capsule, it contains collagenous, elastic fibres and reticular fibres with a large number of blood vessels, lymph vessels, and nerve fibres disseminated throughout (Bhattacharyya, 1972; Jacob & Ziswiler, 1982). Bands of these connective tissues penetrate into the gland forming interfollicular septae, providing support for the secretory epithelium from outside the lobe (Bhattacharyya, 1972; Jacob & Ziswiler, 1982).

1.3.2 Uropygial gland lobules

With only 16% of UGs described anatomically, the information below is based on commonalities observed between families unless otherwise stated. Each lobe of the UG is comprised of many follicles which are lined with secretory epithelial parenchyma. Follicles are present throughout the gland and are considered central, intermediate, or peripheral, depending on their location (Bhattacharyya, 1972). The parenchyma produces secretory products which are deposited into the lumen of each follicle. Secretory products are then relayed through a minute duct in the follicle into a secondary sinus to be stored (Bhattacharyya, 1972; Lucas & Stettenheim, 1972). From the secondary sinuses the secretion is relayed to a primary sinus, and when stimulated by the bill of the bird, the secretion is expelled (Lucas & Stettenheim, 1972; Jacob, & Ziswiler, 1982).

The most common description of follicular structure involves four, well defined epithelial cellular regions (Lucas & Stettenheim, 1972; Jacob, & Ziswiler, 1982; Salibian, & Montalti, 2009; Harem *et al*. 2010). The basal epithelial

layer in a follicle is the germinative layer, followed by the intermediate layer, secretory layer, and finally the degenerative layer (Figure 1.6; Jacob, & Ziswiler, 1982).

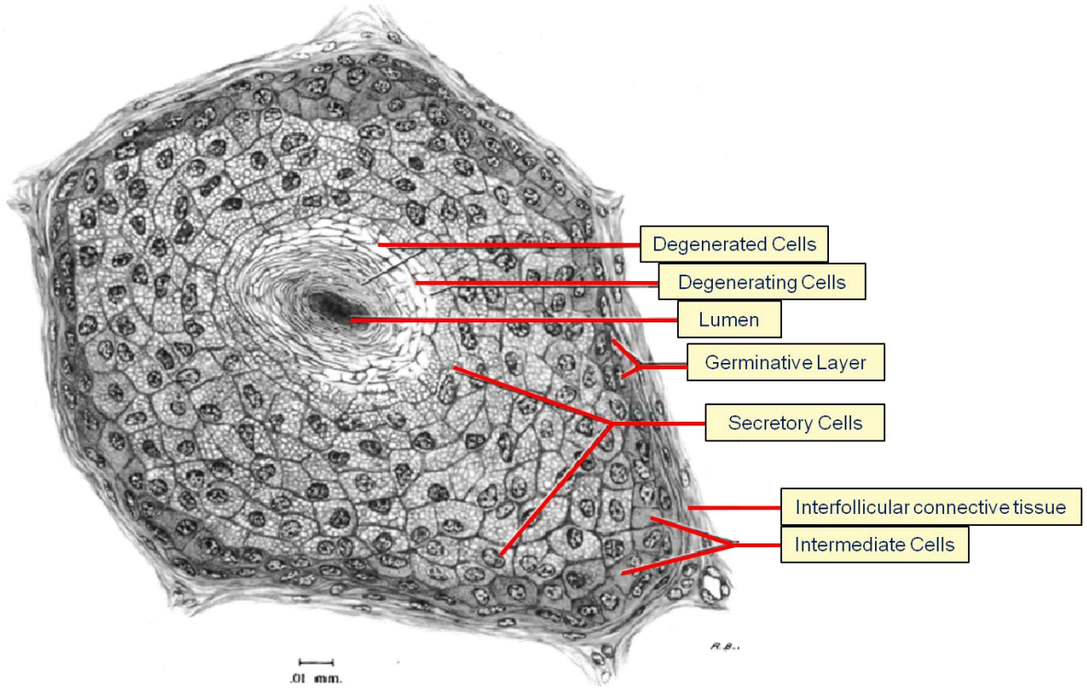

Figure 1.6: Illustration of a follicle from the UG of a Single Comb White Leghorn Chicken . Scale = 0.01mm. (Lucas & Stettenheim, 1972).

The germinative layer is composed of one to two strata of flattened basal cells. It is the chief area of cellular division, and on close inspection different mitotic stages are often visible (Lucas & Stettenheim, 1972; Jacob, & Ziswiler, 1982). The germinative cells are immature in function and become differentiated for secretory functions as they are driven towards the lumen (Jacob, & Ziswiler, 1982). With routine Haematoxylin and Eosin staining this layer is deeply basophilic and is recognised by dark staining of vesicular nuclei and the presence of osmophilic bodies (seen only with electron microscopy), which usually lie close to the nuclei of each cell (Lucas & Stettenheim, 1972).

The next defining layer is the intermediate layer, which is characterised by one to five strata of polygonal cells (Jacob, & Ziswiler, 1982; Salibian, & Montalti, 2009; Harem *et al*. 2010). These cells contain spherical nuclei and a basophilic cytoplasm, rendering them easily stainable (Jacob, & Ziswiler, 1982; Harem *et al.* 2010). In most cases the peripheral follicles have a low number of these layers, whereas central follicles possess more (Harem *et al*. 2010).

Following on from this layer of cells is the secretory layer (Jacob & Ziswiler, 1982). Cells in this layer have differentiated into voluminous polygonal units, one to ten strata thick. Within the cytoplasm of these cells, the Golgi apparatus is extensive and displays numerous sudanophilic granules (Bhattacharyya, 1972; Jacob & Ziswiler, 1982). These granules are stored within the cytoplasm of the secretory cells where they are released on degeneration (Lucas & Stettenheim, 1972).

The degenerative layer is the final, innermost layer where the secretion is liberated into the lumen of the follicle (Harem *et al*. 2010). Here the cells are characterised by pycnotic nuclei – a sign of cellular death – and become irregular in shape (Lucas & Stettenheim, 1972). Cells in this layer prepare for 'death' as they encroach on the lumen of the follicle, hypertrophy occurs, secretion granules coalesce, and finally the cells breakdown, releasing their contents (Lucas & Stettenheim, 1972). Along with products of the secretion, cell fragments and corneous plates are commonly found (Jacob & Ziswiler, 1982; Harem *et al*. 2010).

From the lumen of the follicles the secretory products are moved through the ducts into sinuses where they are stored. The epidermis of the cavities is similar to the epidermis of the follicles (Bhattacharyya, 1972). Between species, Jacob and Ziswiler (1982) found a variety of lengths associated with the ducts and sinuses of the gland. Figure 1.7 illustrates the general structure from follicle through to a primary sinus of a lobe, where a single primary

sinus bifurcates towards the periphery of the gland (Jacob & Ziswiler, 1982). Nevertheless, this figure is highly generalised, as in reality ducts are convoluted, rendering them very hard to follow histologically.

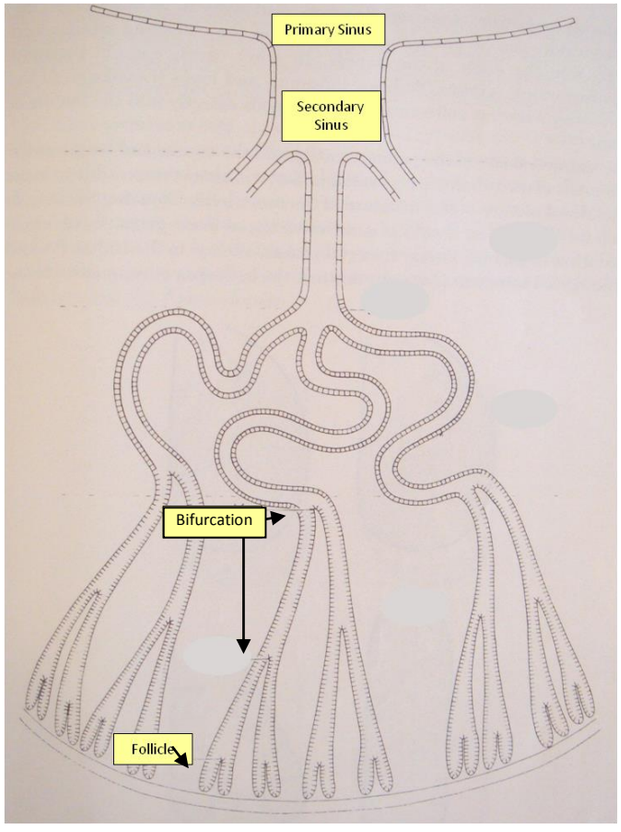

Figure 1.7: Generalised UG structure from follicle through to primary sinus. (Jacob & Ziswiler, 1982).

1.4 Embryology of the uropygial gland in birds

The UG is a specialised epidermal derivative (Bride, 1978), thus it arises from ectoderm in the embryo (Lucas & Stettenheim, 1972). On the dorsal surface of the tail, paired invaginations of ectoderm begin the process of UG development (Lucas & Stettenheim, 1972; Jacob & Ziswiler, 1982). According to Jacob and Ziswiler (1982) this initial step in development occurs on the eight day in both the Zebra Finch (*Poephila guttata*) and pigeon (Columbidae), ninth day in chickens, tenth day in ducks, and on the twelfth day in Budgerigars (*Melopsittacus*

undulatus). As the ectodermal invaginations penetrate deeper, a mesenchymal layer develops at the base from epithelial cells (Jacob & Ziswiler, 1982). As this layer proliferates a simple tubular layer is formed around a central cavity (Lucas & Stettenheim, 1972).

The tubules begin to differentiate into the follicles of the lobes and about the same time the mesenchymal layer produces connective tissue responsible for the development of capsule and interfollicular and interlobular septa (Lucas & Stettenheim, 1972; Jacob & Ziswiler, 1982). Before hatching in most species, lumina develop within the follicles and secretion droplets are formed in the secretory layer of cells (Lucas & Stettenheim, 1972). The timing of papilla development has been found to be species specific (Jacob & Ziswiler, 1982). Homberger (1977) examined a variety of parrots which developed the papilla at differing times, for example a three-day-old black-capped lory (*Lorius lory*) had a fully developed papilla while a ten-day-old red-winged parrot (*Aprosmictus erythropterus*) showed no trace of a papilla. Thus UG development seems to be species specific.

1.5 Blood and nervous supply of the uropygial gland

1.5.1 Blood supply

The UG receives its blood supply from the caudal artery and is drained by the renal portal vein system (Elder, 1954; Jacob & Ziswiler, 1982). The caudal artery divides into branches leading to the dorsal, ventral, and medial regions of the gland's capsule. At these three locations they branch further and enter the capsule wall (Jacob & Ziswiler, 1982). Within the glandular region small anastomosing networks of capillaries form and surround the follicles (Lucas & Stettenheim, 1972). Jacob and Ziswiler (1972) found that the character of this branching was specific at a species level.

1.5.2 The nerve supply

Nerves entering the UG in the chicken (*Gallus* gallus) have a dual origin – medullary and sympathetic – and arise between the first and second caudal vertebrae (Lucas & Stettenheim, 1972). They divide into three separate branches, each innervating skeletal muscle surrounding the area of the gland. Two branches innervate the skeletal muscle of the tail, while the last forms an anastomosis with the sympathetic nerve, becoming the primary uropygial nerve (Lucas & Stettenheim, 1972). Axons have been seen terminating on muscle associated with the papilla as well as in the capsule wall (Elder, 1954; Lucas & Stettenheim, 1972).

1.6 Functions of the uropygial gland

Unlike many other avian and mammalian body features, the UG has not been examined sufficiently for a definitive functional explanation to have been suggested. Because of this, the glands' function remains under debate within the ornithological world. There are four main explanatory hypotheses for the function of the gland: 1) the feather maintenance hypothesis, 2) the water-proofing hypothesis, 3) the communication/health hypothesis, and 4) the defence hypothesis. Each has its own defining characteristics but may only apply to a select few species of birds (Elder, 1954; Jacob & Ziswiler, 1982; Martín-Vivaldi *et al.* 2009; Salibian, & Montalti, 2009).

1.6.1 Feather maintenance hypothesis

Feather maintenance is a fundamental aspect in the life history of birds, especially as feather structure dictates features such as flight and thermoregulation. The feather maintenance hypothesis suggests the UG's secretion maintains the keratin in the feathers, which keeps them flexible, pliable, and in a good condition (Stettenheim, 2000). An investigation by Elder (1954) illustrated this effect by removing the UG in five Red-head (*Aythya americana*) and five Shoveller (*Spatula clypeata*) ducklings. He showed that the plumage of the birds became dull, dry, and unmanageable as the ducklings grew, though preening behaviour appeared normal. The birds did not

survive as long as the controls (birds with intact UG's) because their plumage was easily soiled and roughened, exposing them to injury and disease (Elder, 1954; Bhattacharyya, 1972).

However, the UG is a vestigial feature in some avian species (e.g. Stuthionidae, Columbidae, Ardeidae, and Psittacidae) (Menon, & Menon, 2000; Salibian, & Montalti, 2009) and how these birds maintain their feathers has not been explained. Powder-down feathers have been hypothesised as an alternative way to maintain other body feathers (Menon & Menon, 2000). Powder-down feathers are either found in patches on the body (e.g. herons – Ardeidae) or are scattered throughout the plumage (King & McLelland, 1985). The barbs at the tips of the rachis constantly break away, forming minute particles of keratin which coat the plumage (King & McLelland, 1985; Menon & Menon, 2000). The fine powder that coats the plumage is speculated to replace the 'feather maintenance' function in birds lacking UG's e.g. members of Psittaciformes and Pelecaniformes (Menon, & Menon, 2000; Montalti, & Salibian, 2000). However, some species of birds, such as members of the Ardeidae, possess small UGs as well as powder-downs (Wetmore, 1920), which may suggest that the UG or the powder-down feather may have a different function than feather maintenance.

1.6.2 Water-proofing hypothesis

The waterproofing hypothesis was postulated more than a century ago and is surrounded by much controversy. Salibian and Montalti (2009) hypothesised that if the UG secretion was used for waterproofing feathers then aquatic birds should have larger UGs than terrestrial birds. When they compared the UGs of aquatic and terrestrial birds, there was no significant difference in the size or, degree of development of the gland and the bird's relationship with water. Therefore, they concluded that the UG played no significant role in waterproofing.

However, Salibian and Montalti (2009) did not take into account the possibility that the chemical compounds of the secretion affected water-proofing. For example, aquatic birds could have smaller or similar sized glands but

having more concentrated secretions. In contrast to the above study, Giraudeau, *et al.* (2010) provided evidence that the gland is used in waterproofing. When access to the UG was temporarily (three months) prevented in a group of experimental birds, their plumage showed lower water-repellence compared to control birds. This result, however, may have ensued from the deterioration of the plumage condition (hypothesis 1), thus disrupting the micro-structure of the feathers, which is most commonly proposed as a water-proofing strategy.

1.6.3 Intraspecific communication/health hypothesis

Seasonal differences within the chemical composition of the secretion have led some scientists to suggest an intraspecific communication function for the gland. Kolattukudy and colleagues in 1987 produced evidence of a sex pheromone in the female mallard's UG secretion. They showed that the composition of the secretion changed seasonally and that just before the mating season the secretion contained a chemical which actively attracted males. The blue petrel (*Halobaena caerulea*), described by Mardon, Saunders, and Bonadonna (2011) is another bird which utilises uropygial gland secretions in olfactory communication. This monogamous, burrow-nesting species is a representative of the family Procellariiformes, a group of seabirds whose olfactory neuroanatomy is highly developed (Mardon, Saunders & Bonadonna 2011). Mardon, Saunders, and Bonadonna (2011) found that the UG of these birds plays a fundamental role in the development of individual odour and thus conspecific recognition.

Another aspect of this hypothesis looks at cosmetic colouration. Greater flamingos (*Phoenicopterus roseus*) incorporate carotenoids into their uropygial oil and can alter plumage colouration without a moult ever occurring (Amat *et al.* 2011). This is thought to function in sexual selection, whereby the more brightly coloured individuals are seen to be more 'attractive'. The more carotenoids that are applied to the plumage the brighter the greater flamingo's plumage becomes and thus they become more attractive to the opposite sex (Amat *et al.* 2011). In the

late 1920's the UG secretion was investigated for its anti-rickets effects (Hou, 1929). However, the UG anti-rickets hypothesis was dejected when Rawles (1960) found that the UG was not required for calcium metabolism.

1.6.4 Defence hypothesis

The evolution of uropygial secretions employed as a defence strategy involves toxic, unpalatable, and foul smelling chemicals that the bird applies to its plumage in order to ward off predators and microbial parasites (Martín-Vivaldi *et al*. 2009). The use of the secretion in predator avoidance is best illustrated in the genera *Pitohui* (Dumbacher *et al.* 1992). The Hooded (*Pitohui dichrous*) and Variable (*Pitohui kirhocephalus*) Pitohuis produce a different uropygial secretion than any other bird so far reported (Dumbacher *et al.* 1992). Their insectivorous diet (Jonsson *et al*. 2008) incorporates the beetles of the genus *Choresine* (Rajchard, 2010), which harbour batrachotoxins – some of the most potent poisons known to man (Hagelin & Jones, 2007). The UG incorporates batrachotoxins from the diet into the secretion, and when applied to the plumage becomes toxic to predators (Rajchard, 2010).

Defence is not only required against predators, but also against microbial pests. With experimental removal of the UG, levels of microflora on the feathers increases in some birds, thus supporting the idea of the secretion having an antimicrobial function (King & McLelland, 1985; Moller, Erritzoe, & Rozsa, 2009). An investigation by Shawkey, Pillai, and Hill (2003) examined the effects of UG secretion on the growth rate of bacteria isolated from the feathers of wild house finches (*Carpodacus mexicanus*), and found that the secretion inhibited the growth of strong feather-degrading bacteria. As well as bacteria, Moyer *et al.* (2003) showed how uropygial gland oil from rock doves (*Columba livia*) increased the mortality of lice (*Columbicola columbae* and *Campanulotes bidentatus compar*) *in vitro* by exposing lice to feathers with and without uropygial gland oil. They discovered that lice exposed to feathers with uropygial oil suffered higher mortality than lice not exposed to uropygial oil.

1.7 Why should we investigate the uropygial gland of New Zealand birds?

The hihi is the only representative of an endemic New Zealand family, the *Notiomistidae* (Driskell, 2001; Driskell et al. 2007) that predominantly resides in forest (Figure 1.8). It is classified by the IUCN as vulnerable and by DOC (Department of Conservation) as nationally endangered (Taylor, Castro & Griffiths, 2005; IUCN, 2010*).* Hihi were originally distributed throughout the North Island mainland, Great Barrier Island, Hauturu (Little Barrier Island), and Kapiti Island. In 1873, hihi populations were reducing in numbers and by the 1880's populations of hihi had declined to a single surviving population on Hauturu (Taylor, Castro, & Griffiths, 2005). Many factors have led to the hihi's decline. Hihi construct their nests in cavities which subject them to threats of tree-scaling predators and forest clearance (Makan, 2006). Therefore, introduction of the exotic ship rat (*Rattus rattus*), together with the destruction of habitat as well as possible diseases, have all caused the extinction of hihi from the mainland.

New Zealand has two endemic Meliphagidae species – the tui (*Prosthemadera novaeseelandiae*) (Figure 1.9) and the New Zealand bellbird (*Anthornis melanura*) (Figure 1.10). Hihi was once a part of the Meliphagidae until genetic investigations classified it to its own monotypic family. The tui population in New Zealand is generally declining and has succumbed to pressures of human colonisation (Diamond & Veitch, 1981). Similarly, the New Zealand bellbird population now reflects areas of remaining native forest throughout the country – areas which have been dramatically reduced (Baillie, 2011).

In order to continue to unveil the connection between structure and function of the UG, the examination and comparison of the morphology of the UG of these three New Zealand birds was undertaken (Chapter 3). The recent alteration in the taxonomy of hihi provided me with an opportunity to compare its UG with that of other New Zealand passerines – in particular bellbirds, tui and saddleback – to test whether the structure of the gland is related to their phylogeny. The saddleback (*Philesturnus carunculatus*) (Figure 1.11), in the family Calliadae, is closer taxonomically to Notiomystidae than to Meliphagidae.

Figure 1.8: Photograph of a male hihi. Photograph taken by Isabel Castro.

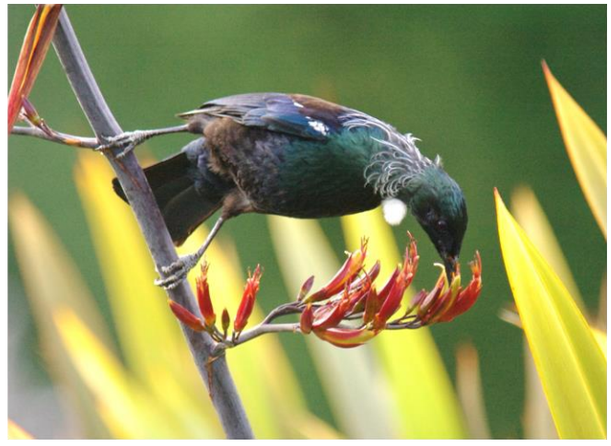

Figure 1.9: Photograph of a New Zealand tui. Photograph taken by Isabel Castro.

Figure 1.10: Photograph of a New Zealand bellbird. Photograph taken by Isabel Castro.

Figure 1.11: Photograph of a New Zealand saddleback. Photograph taken by Isabel Castro.

Figure 1.12: Photograph of a brown kiwi. Photograph taken by Jay Bent.

Kiwi, the smallest of the ratites, are endemic, flightless birds (Figure 1.12) and are primarily nocturnal forest dwellers (Higgins *et al*. 2001)**.** There are five separate species of kiwi that reside within New Zealand, each possessing its own defining characteristics (Burbidge *et al.* 2003). The most abundant of these is the brown kiwi (*Apteryx mantelli*), which are relatively sedentary birds that occupy defined home-ranges, and have a mating system ranging from monogamy to cooperative breeding (Ziesemann *et al*. 2011). In high density populations the home ranges of many birds overlap. For example, on Ponui Island, pairs or groups of birds often share – both sequentially and simultaneously – diurnal roosting sites and nests (Ziesemann, 2011). This pattern of burrow use offers an opportunity for parasites to build up and be transmitted between hosts. Indeed, Ponui Island birds carry large ectoparasite (*Ixodes anatis*) burdens (Heath, 2010).

It is thought that brown kiwi most commonly produce one to two eggs per clutch and can have up to three clutches in one season. Contrary to many other avian species, the male is generally responsible for incubation which may last for 74-84 days (Ziesemann *et al.* 2011). Again this long nest occupancy exposes these birds to heavy ectoparasite infestations. The kiwi UG is relatively large compared to the bird itself (Chapter 2), suggesting that this gland may have an important role. It is possible that chemicals within the UG secretion may provide the skin

with anti-parasitic effects. When applied to the plumage these chemicals may aid in preventing large ectoparasite loads on the birds' plumage. Not only may the secretion have anti-parasitic effects, it may also function to maintain populations of commensal and/or symbiotic micro-organisms that aid in keeping the plumage in good condition. Because the UG secretion is composed of oils, evaporation from the plumage surface takes a considerable amount of time (Haribal *et al.* 2005). Thus ectoparasites may be repelled by the secretion while favourable micro-organisms might be encouraged to grow.

1.8 Aims of the study

This study aims to improve our understanding of the structure and function of the UG of birds by achieving the following aims:

- → 1) To describe the morphological and histological structures of hihi, tui, New Zealand bellbird, saddleback and kiwi UGs, by examining the glands of dead birds (chapters 2 and 3).
- → 2) To investigate whether or not sex or season of hihi affects the cellular makeup of the the UG (chapter 4).
- → 3) To examine the behaviour of the ectoparasite, *Ixodes anatis* (kiwi tick) by recording their taxic response to the UG secretion of brown kiwi UG (chapter 5).

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Chapter 2: The Uropygial Gland of Kiwi (*Apteryx* spp): Morphology and Pathology.

ABSTRACT

The uropygial gland is a prominent feature in avian anatomy but there is little information on its structure. In this study uropygial glands of 29 brown kiwi (*Apteryx mantelli*) were examined grossly and the uropygial glands of eight brown kiwi, five great spotted kiwi (*A. haastii*) and one Haast tokoeka (*A. australis*) were examined histologically.

Kiwi uropygial glands were found to be bilobar and possessed eight primary sinuses, each opening through its own orifice in the gland's papilla. Primary ducts were of the compact type and the gland's capsule had branches of connective tissue extending internally to form interfollicular septae. Interfollicular septae were thicker in some areas and caused follicles to be grouped into discrete lobules. Van Gieson staining revealed tendon-like connective tissue connecting the underlying striated coccygeal muscle to the capsule of the uropygial gland. It is thought that this feature may be used in controlling the expulsion of secretion from the gland. The follicular epithelium was similar to that in other species and consisted of four cellular layers: germinative, intermediate, secretory, and degenerative although the degenerative layer was barely discernible in most sections. More cells contributed to the follicular epithelium in male kiwis than in female uropygial glands. This suggests that male kiwi may produce more secretion than females.

The uropygial glands of one brown kiwi, two great spotted kiwi and one Haast tokoeka exhibited pathological changes. Atrophy of the uropygial gland was seen in two birds in poor condition in which follicles had reduced epithelial thickness, expanded lumina, and a lack of secretory products. One bird showed pustule formation which affected the uropygial papilla and ballooning degeneration occurred in the glandular epithelium. In another

individual localised uropygial adenitis was found in the ventral sinus of the gland. Keratinaceous necrotic debri containing Gram-positive cocci occupied the entire sinus and there was a mixed inflammatory cell infiltration of the surrounding interstitium.

The uropygial gland of kiwi was different from all other uropygial glands described thus far; not only were there differences in structure but differences between the sexes were also observed histologically. This study adds to the sparse array of histological investigations completed on these glands to date and provides a starting point for future investigations into the evolution and structure of the glands in other species.

2.1 Introduction

The uropygial gland (UG), also known as the oil gland, rump gland or preen gland (Jacob & Ziswiler, 1982; Sadoon, 2011) is one of only three integumentary glands found in birds. This is in marked comparison to other vertebrate groups, such as the reptiles and mammals, which possess a range of differing glands over their body surfaces (Quay, 1972). The morphological characteristics of the UG are quite different between species and even between individuals (see Figure 1.3; Salibian & Montalti, 2009). The shape, size, and uropygial circlet feather arrangement have previously provided the gland with some taxonomic significance (Johnston, 1988; Salibian & Montalti, 2009). Histologically, the UG corresponds to the mammalian sebaceous gland because of its holocrine structure (King & McLelland, 1985; Sara, *et al.* 2006). Homology between the UG and the structure of reptilian glands is uncertain (Elder, 1954). However, links between the UG secretion and that produced by some reptilian integumentary glands are believed to have 'ancestral cytogenetic relationships' (Quay, 1972).

Holocrine glands are a type of exocrine gland comprised of differentiating, volumating cells which degenerate towards the lumen, thereby releasing the secretion (Cormack, 1987; Kanitakis, 2002). The arrangement of cells in a holocrine gland is structurally complex. This arrangement of cells allows for storage of the secretion within sinuses, and when stimulation occurs (either mechanically or by nervous innervation), the secretion is released and relayed through a duct system towards the skin's surface (Marieb & Hoehn, 2007). Studies to date have described four key layers of cells within each UG follicle: 1) germinative layer; 2) intermediate layer; 3) secretory layer; and 4) degenerative layer (see Figure 1.6; Jacob & Ziswiler, 1982; Sawad, 2006; Sadoon, 2011).

Only 0.2% (20/10,000) of known bird species have had their UG investigated histologically. Generalities in structure have been observed between them, such as the follicle cell layers described above, but differences between species are evident in many other aspects of UG histology, such as follicle layout around the primary cavity (Jacob & Ziswiler, 1982). Therefore, many features of this gland remain a mystery, for example its innervation, mode of

secretion and expulsion from the papilla are still not well understood. Kiwi are part of an ancient radiation of birds and are grouped within the ratites. Collectively ratites and Tinamiformes make up the palaeognathae lineage (Sibley & Frelin, 1972; Cooper *et al.* 2001) and within this group it is known that only kiwi and tinamous possess UGs as adults (Johnston, 1988). Obtaining information on the structure, histology and innervation of the kiwi UG will provide the first detailed insight into a species from the palaeognathae lineage.

2.1.1 Study aims

This chapter aims to provide the first detailed description of the morphology of the UG of kiwi, including its gross anatomy and histology and also provides some examples of pathological changes that were observed.

2.2 Materials and methods

2.2.1 External morphometrics

Photographs and external measurements of the UG of 15 male and 14 female wild brown kiwi (*Apteryx mantelli*) from Ponui Island (1770 ha; 36 55', 175 11'E) were taken in February/March 2008 and again in March 2009; and six females (one in 2008) and three males (one in 2008) were sampled once by I. Castro and S. Cunningham (permits: NZ DoC AK-29244-FAU; Massey University Animal Ethics 07/97). They recorded the following measurements: UG base length (cranio to caudal part of kiwi body) and width (side to side of kiwi body); UG height from the point where the UG met the body to its tip. To determine an index of volume, the cone volume formula was used: $v = 1/3*\pi*r^2*h$; where $\pi = 3.14159265$; r = ((maximum length + maximum width)/2)/2) and h = UG height. Colour of the UG secretion was noted.

2.2.2 Histological and pathological examination

Eight dead brown kiwi (Table 2.1), five great spotted kiwi (*Apteryx haastii*; Table 2.1) and one Haast tokoeka (*Apteryx australis* var. Haast) in fresh condition that died from a variety of causes were sourced from birds

submitted to Massey University's Wildlife Health Centre from March 2011 to June 2012. I dissected UGs from carcasses with a tissue margin sufficient to include the surrounding fat and connective tissue. This decreased the chance of accidentally slicing the gland in the wrong orientation. After the gland had been removed from the bird it was fixed in 10% neutral buffered formalin. Each gland was sectioned sagitally into three slices (Figure 2.1). Initially, a section was made down the midline of the gland between the papilla (Figure 2.1). One side of the gland was then used to produce a midline section, and two lateral sections (lateral 1 and 2).

Table 2.1: Sample sizes for histology aspect of investigation (excludes birds used for pathology).

Species	**Male**		**Female**	
	Juvenile	**Adult**	**Juvenile**	**Adult**
Brown	2	2	0	3
Great Spotted	0	1	2	0

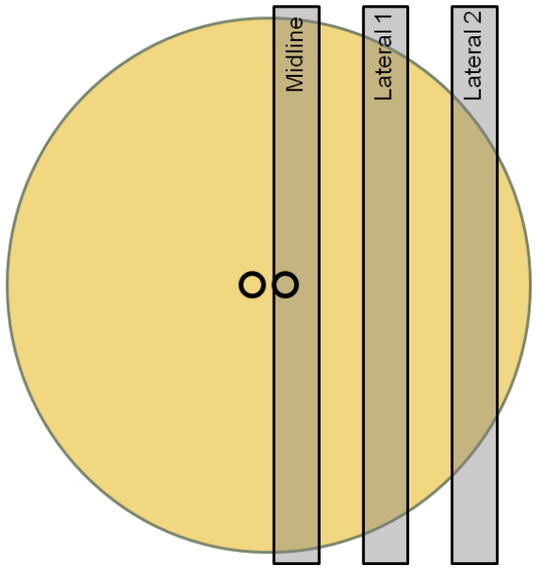


Figure 2.1: Model illustrating histological sections produced from each UG - the two small circles in the middle represent dorsal view of papilla. Illustrations by author.

Each section was embedded in Leica Histo Embedder (Leica Paraplast® Tissue Embedding Medium (Melting Point 56ºC)) and cut at 4µm using a Leica RM2235 Manual Rotary Microtome with a S35 Feather Microtome Blade (stainless steel). Sections containing bone were decalcified before being cut to avoid tearing the glandular region of tissue. Decalcification of sections was undertaken in osteomoll ® (Merck) overnight prior to processing. Each slide (Menzel-Glaser Superfrost PLUS Slides 76 x 26mm Ground edge 90º) was then routinely stained with Haematoxylin and Eosin (H&E) and was coverslipped wet via a xylene bath (Leica CV 5030 robotic coverslipper; Coverslips = Menzel-Glaser 22 x 50 No. 1; Mountant = Entellan ® rapid mounting medium for microscopy). Once dry, they were examined under a light microscope with measurements of the glandular area being made with a Colourview digital camera and an AnalySIS 5 software-image analysis system.

To investigate secretory cell products and the structure of the gland, histochemical stains were applied to each section. Masson's Trichome and Van Gieson stains identified connective tissue and muscle and Sudan Black identified lipid. Gram stains were used to investigate bacteria when present. Techniques for each stain followed procedures in Bancroft and Gamble (2008).

2.2.2.1 Histomorphometrics

For each section I placed a longitudinal transect representing the maximum glandular diameter was made at 1.25x magnification (Figure 2.2). I measured the follicles along this transect at 20x magnification. The mean follicular diameter and mean luminal diameter of each follicle were calculated. The number of cells contributing to the follicle cell layer and their degree of vacuolation (see section 2.3.3) were recorded.

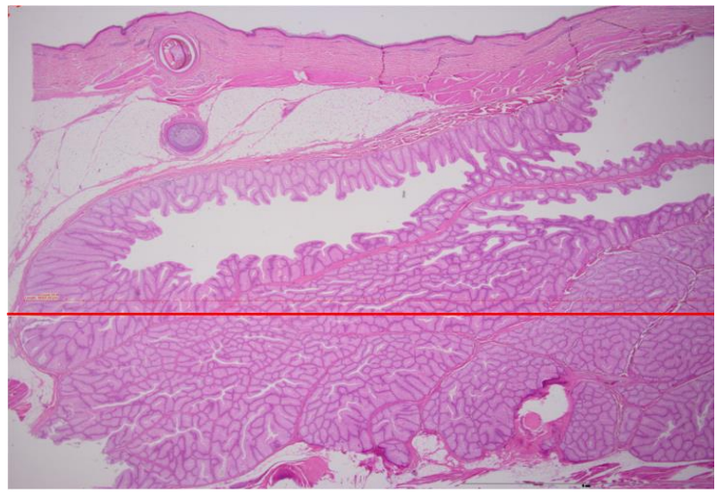


Figure 2.2: Photomicrograph of the UG of a brown kiwi showing the longitudinal transect of maximum glandular diameter. H&E scale bar = 5mm. Photograph by author.

2.2.3 Statistical analysis

A Mixed Model Repeated Measures Analysis (MMRMA) was carried out using SPSS 20 (2011) to investigate the effect of age (juvenile, adult) and sex (male, female) on UG volume for the sample of 29 wild birds. A model was built where bird was the repeated measure, and volume (year 1 vs. year 2) the dependent variable; age and sex were between subjects factors and tarsus measurement was used as a covariate to control for differences in size between the sexes (male brown kiwi are generally smaller than the females). UG volume was not normally distributed (Shapiro-Wilkinson test (S-WT) = 0.87; P = 4.8E-6) so the data was transformed using log 10 to conform to normality (S-WT = 0.99; P = 0.90).

Unpaired t-tests were used to examine the effect of sex on the mean length, width, and depth of the UG. Paired t-tests were used to detect significant differences in mean follicular diameter, luminal diameter, number of cells in a follicle cell layer, number of germinative cells in a follicle cell layer, number of intermediate cells in a follicle cell layer, and number of secretory cells in a follicle cell layer between the middle and outside of the UG. Unpaired t-

tests were also used to assess the effect of species (North Island Brown, Great Spotted) and sex (male, female) on the histology data above.

All statistics are given to three decimal places. A probability of ≤0.05 was taken as significant except when several tests were made on various measurements of the UG. For these, a Bonferroni corrected p value was used calculated as 0.05/number of tests performed.

2.3 Results

2.3.1 Gross anatomy of the kiwi uropygial gland

The gland was large in size compared to other birds and had an unusual location immediately dorsal to the cloacae (Figure 2.3) and surrounding the coccygeal bone (Figure 2.4). The UG was bipartite (Figures 2.5 & 2. 11) with individual variation in shape and size of the gland. The UG volume ranged from 4.77 to 0.35 cm^3 in 2008 (Median = 1.57); and from 0.27 to 2.97 cm^3 in 2009 (M = 0.94). There was a significant difference in volume between years with UGs in 2008 having significantly larger volumes than in 2009 (paired t-test t = 5.8; df = 28; P <0.0001; Figure 2.6). After correcting for size, age (MMRMA, t = 0.102; df =25.16; P = 0.92) and sex (MMRMA, t = 1.15; df =27.54; P = 0.26) were not good predictors of UG volume.

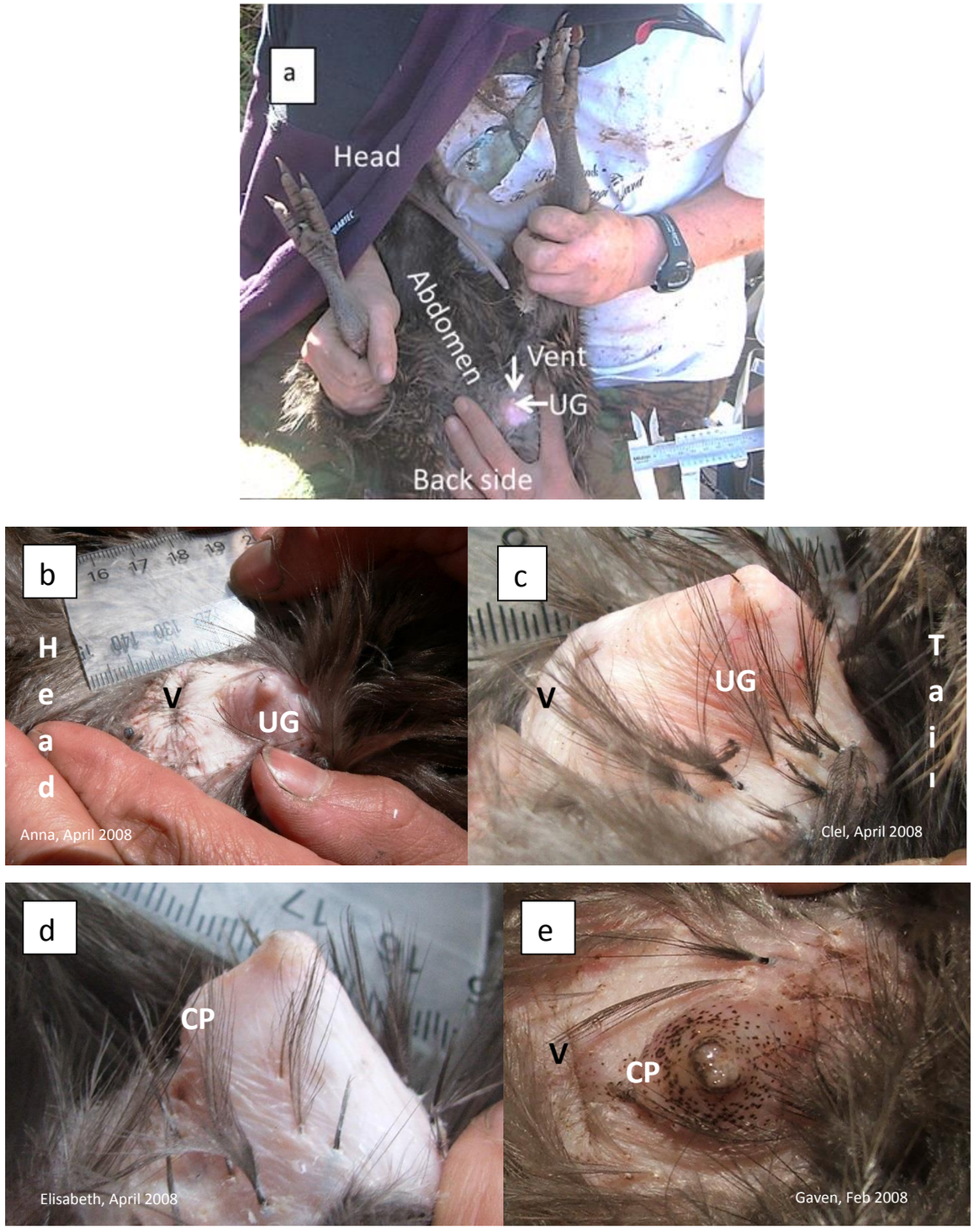


Figure 2.3: Brown kiwi <u>Apteryx mantelli</u> UG. a) Position of the bird for measurements and photographs; b and e: photos taken from above; c and d: photos taken from the side. For b-e, the head of the birds is located to the left of the photographs and the backside to the right. b)Location of the UG in relation to the vent (cloaca), V = vent; UG = Uropygial gland. Notice the presence of two (b) and one (c) feathers respectively at the apex of the gland and the variation in the proximity of the gland to the vent; in b the vent is separated from the UG by about 3mm while in c the vent surrounds the UG. c and d) Un-pigmented UG showing a prominent forward extension of the gland that was named cartilaginous protuberance or CP, also visible in e. e) UG showing high pigmentation. Photographs by Isabel Castro

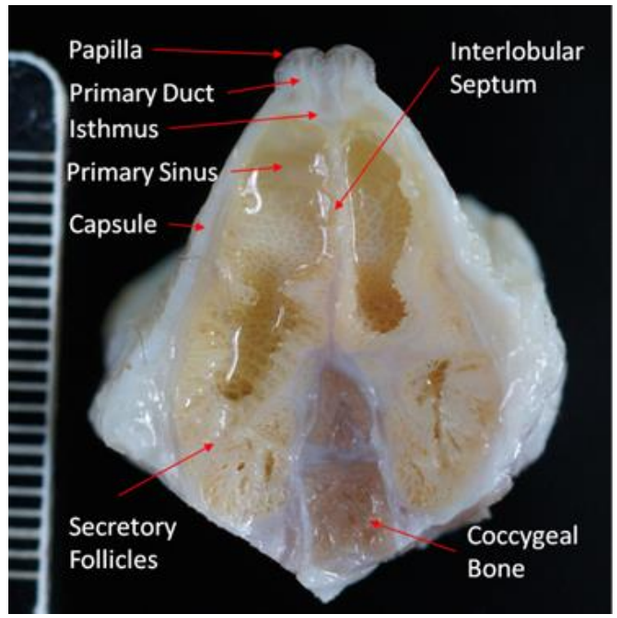


Figure 2.4: Brown kiwi UG anatomical organisation. Mid transverse section. Scale graduations = millimetres. Photograph by Maurice Alley.

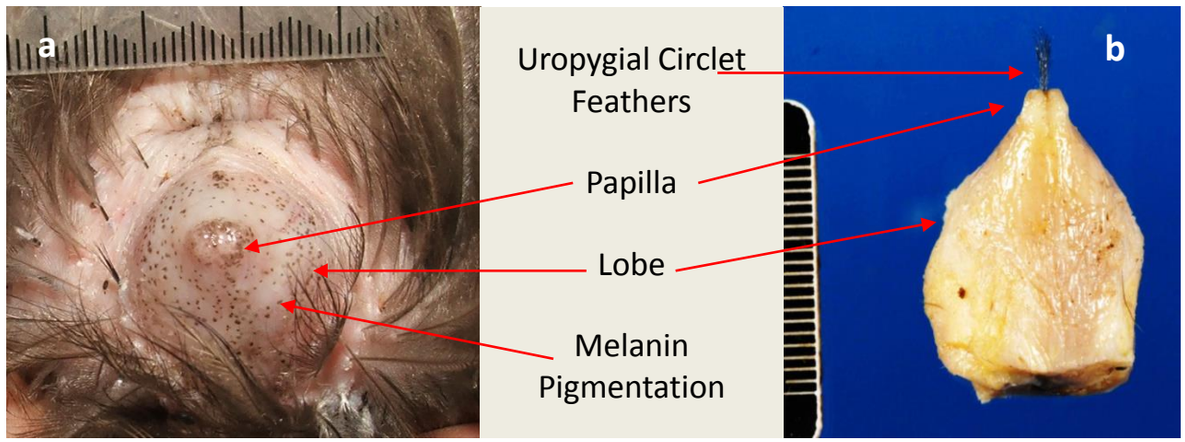


Figure 2.5: Photographs of brown kiwi UG: a) showing dark pigmentation spots on its surface due to melanin pigments; b) ventral view showing two lobes, a papilla, and uropygial circlet feathers. Scale = millimetres. Photographs taken by a) Isabel Castro and b) by author.

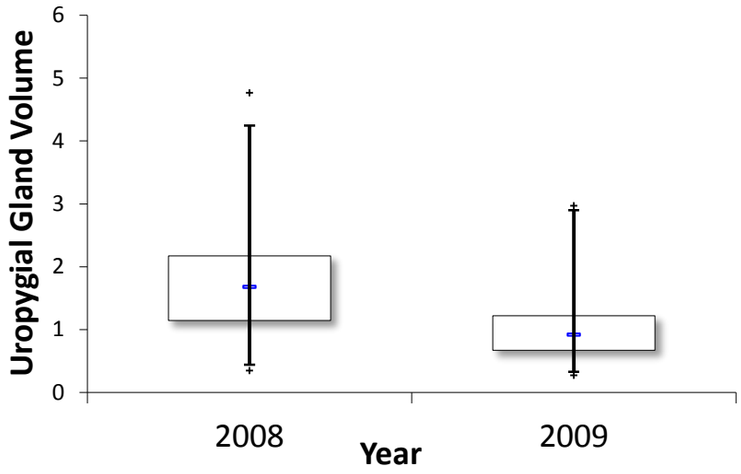


Figure 2.6: Relationship between UG volume in the two years of the study. The short line within the box represents the median of the UG volume. The bottom and top edges represent the 25th and 75th percentiles. The "whiskers" extend to the 5th and 95th percentiles. The minimum and maximum values in the sample are indicated with a '+' sign.

The bilobed papilla protruded from the two lobes of the gland and in most birds examined the transition from lobes to papilla was gradual with no clear division being visible (Figure 2.5). Up to two feathers were present at the top of the gland (Figure 2.5 & Figure 2.7a). The pigmentation of the UG consisted of freckling of the skin and was very prominent in some birds (Figures 2.3c & 2.5). When comparing the pattern of pigmentation of individuals between sampling periods it remained constant so this characteristic could be used for identification. Pigmentation was significantly (Chi square = 10.0; df = 2; $P < 0.05$) more common in females (10 of 18) than males (5 out of 17; Figure 2.8). In addition, some birds without pigmentation on the body of the UG had the tip pigmented; this was also more common in females (5/9) than males (1/12). The UG secretion had four different colorations: transparent, pale gold, gold, and ivory; half of the birds secretions were one colour the first year and a different one the second (Figure 2.7b). The most common colour in both years was pale gold (14/30 and 18/34) followed by gold (12/30 and 7/34). The secretion was usually transparent but in two birds it was turbid at one of the sampling periods.

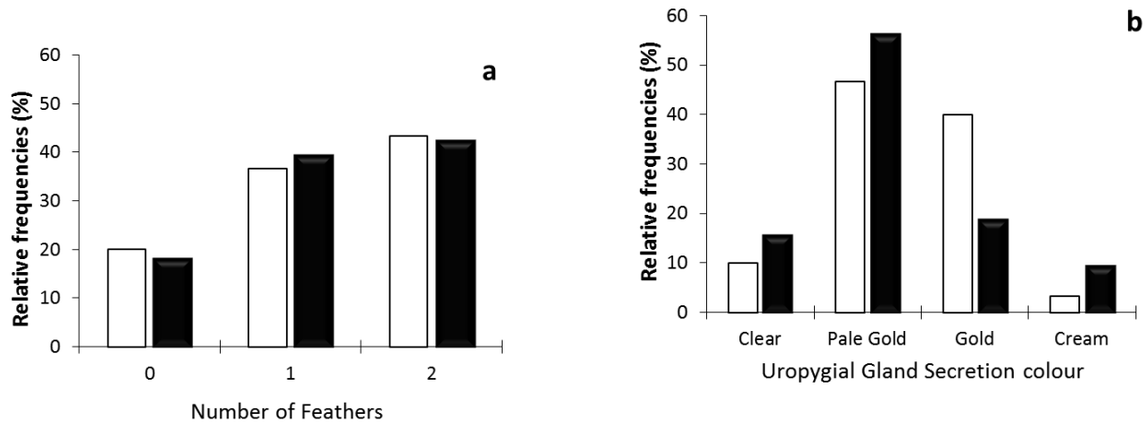


Figure 2.7: Characteristics of the kiwi UG and its secretion. a) Number of feathers present on the UG. b) UG secretion colour. White bars = 2008; black bars = 2009.

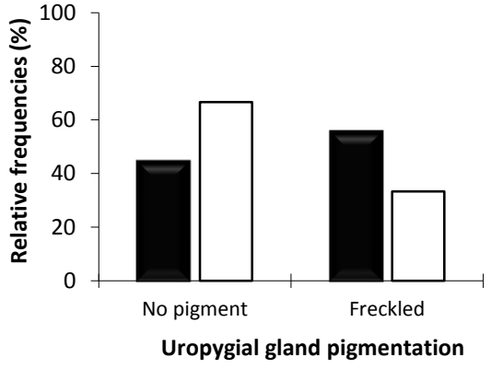


Figure 2.8: Percentage of males and females presenting pigmentation of the UG. Black = females; whilte = males; n = 13 females, 15 males.

2.3.2 Microscopic anatomy of the kiwi uropygial gland

The UG of seven brown and three great spotted kiwi were used. The UG of the remaining four birds showed pathological changes that are discussed in section 2.3.4 below. The uropygial glandular elements were bound together by a connective tissue capsule. In some kiwi the dorsal surface of the UG contained melanin pigment which caused it to appear spotted or very dark (Figure 2.5). The capsule consisted of thick, branched, and linked collagen fibres visible with a Massons Trichome stain (Figure 2.9). Outside the capsule and between the integument were bands of smooth muscle (Figure 2.10). Bands of collagen and elastic fibres formed the interfollicular septae,

penetrating into the glandular area (Figure 2.9). Small nerves penetrated both the capsule and interfollicular septae (Figure 2.9) and may be related to expulsion of the secretion from the gland. Striated muscle underlying the gland was shown to form tendon like structures connected to the capsule of the UG in some instances (Figure 2.11).

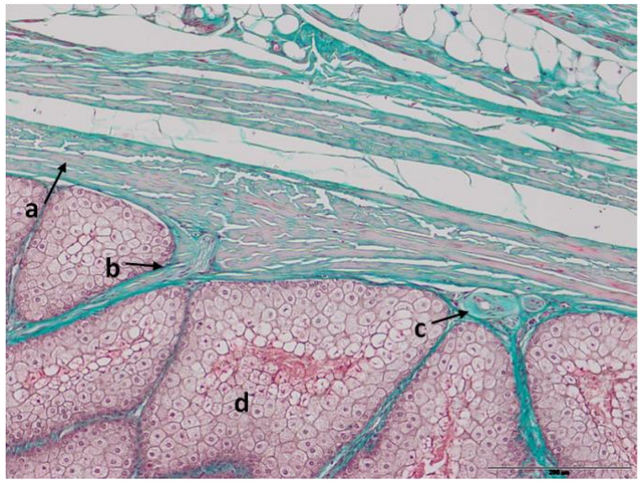


Figure 2.9: Photomicrograph of brown kiwi UG: a) capsule; b) branching of interfollicular septae into glandular area c) nerves; d) follicle. Massons Trichome scale bar =200um. Photograph taken by author.

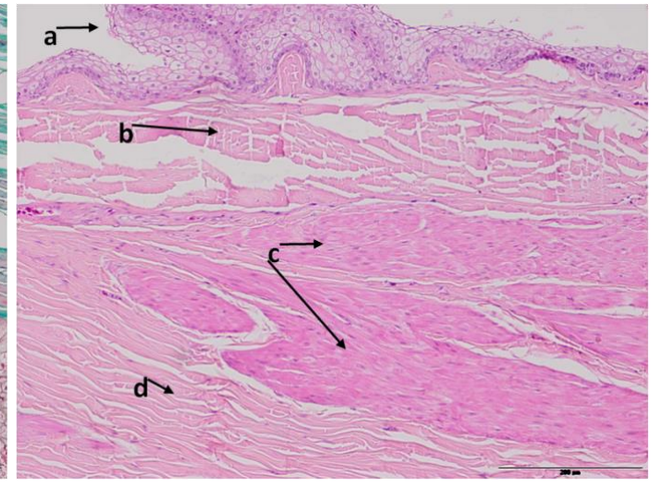


Figure 2.10: Photomicrograph of brown kiwi UG: a) glandular area; b) capsule c) smooth muscle; d) dermal collagen. H&E scale bar =200µm. Photograph taken by author.

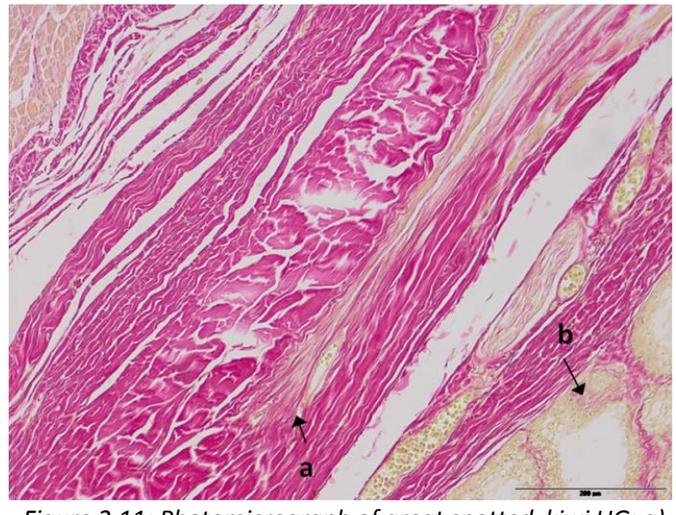


Figure 2.11: Photomicrograph of great spotted kiwi UG: a) tendon formed with capsule of UG; b) secretory area. Van Gieson stains muscle pink and connective tissue yellow; scale bar =200µm. Photograph taken by author.

Beneath the UG papilla was a connective tissue isthmus through which the primary ducts ran (Figure 2.4). The kiwi primary ducts were of the compact type (see chapter 1) where connective tissue surrounding the ducts was dense, causing them to be narrow (Figure 2.13). There were no cross-channel connections between primary ducts, therefore the secretion would be expelled through individual orifices (Figure 2.13). Each lobe possessed four distinct primary sinuses and each was associated with its own primary duct (Figures 2.12 & 2.13). In total therefore, there were eight orifices (duct openings) in the papilla. Each of the two lobes in the kiwi UG was elongated and sat dorsal to the coccygeal bones (Figure 2.4). The glandular tissue predominated at the base of its associated sinus (Figures 2.4 & 2.12). Sudan Black staining revealed the presence of dense sudanophilic lipids within the lumen of many follicles (Figure 2.14).

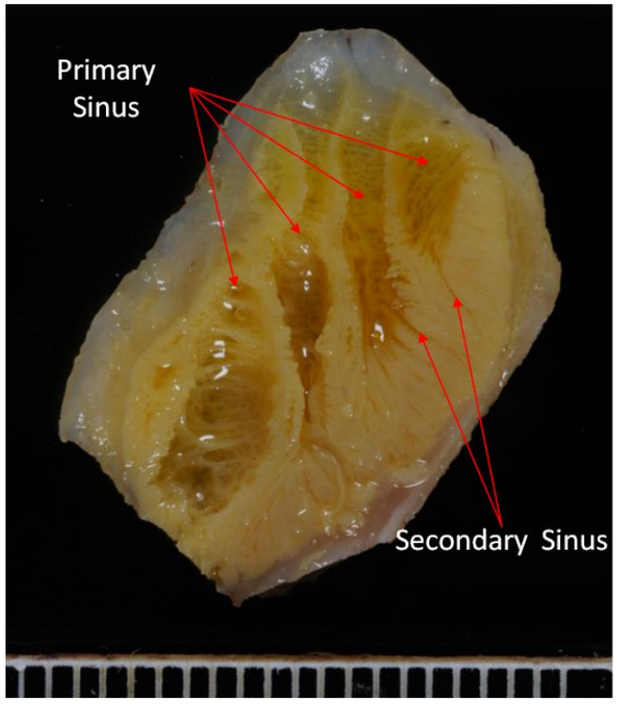


Figure 2.12: Photograph of the inside of a lobe of a brown kiwi UG. Four primary sinuses and some secondary sinuses are clearly visible. Lateral sagittal Section. Scale graduations = millimetres. Photograph by author.

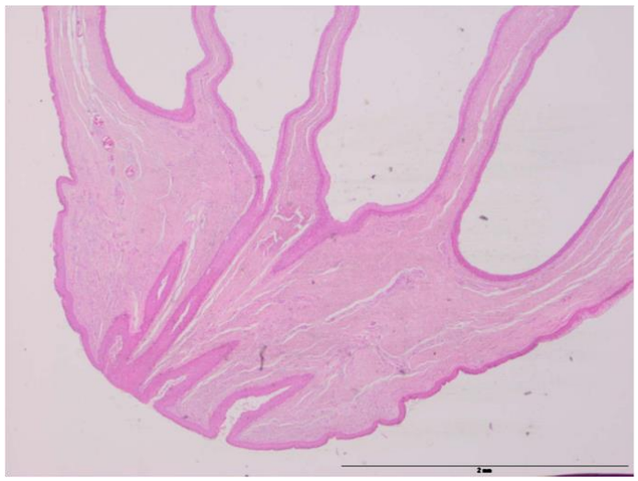


Figure 2.13: Photomicrograph of great spotted kiwi UG midline section. Showing compact type of four primary ducts, each associated with their own sinus. H&E scale bar = 2mm. Photograph by author.

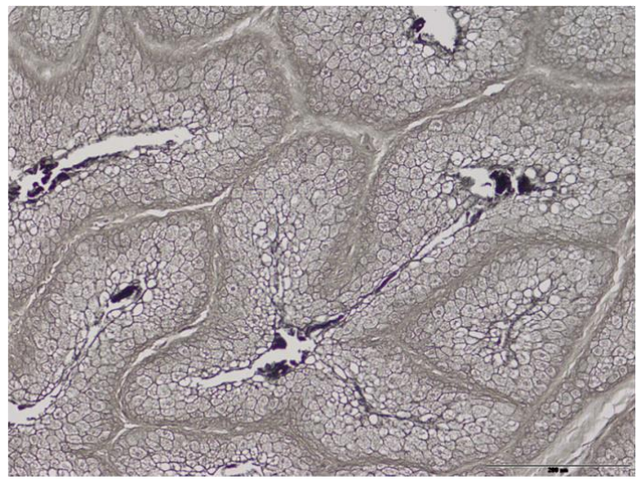


Figure 2.14: Photomicrograph of brown kiwi UG lateral section. Showing lipids in the secretion staining strongly positive with Sudan Black, scale bar = 200µm. Photograph by author.

2.3.3 Histology of the kiwi uropygial gland follicles

Three separate cell types were identified which were classified as 'germinative', 'intermediate', and 'secretory' (Figure 2.15). The germinative layer of cells consisted of those which were densely stained with a uniform, eosinophilic cytoplasm indicating that very little secretion had accumulated (Figure 2.15; Jenik, Fisch, & Goodridge, 1987). These cells most probably correspond to the germinative layer described by Jacob & Ziswiler (1982). Intermediate cells were those which had small vacuoles of secretion within their cytoplasm (Jenik, Fisch, & Goodridge, 1987) and were distinguished from the secretory cells by their centrally located nuclei (Figure 2.15). The secretory cells were clearly vacuolated and all the cytoplasm was filled with secretory products (Figure 2.15). In these cells nuclei were displaced peripherally. These cells correspond to the secretory and degenerative areas described by Jacob and Ziswiler (1982), although degenerative cells were much flatter, smaller and more disrupted than the underlying secretory cells (Jenik, Fisch, & Goodridge, 1987).

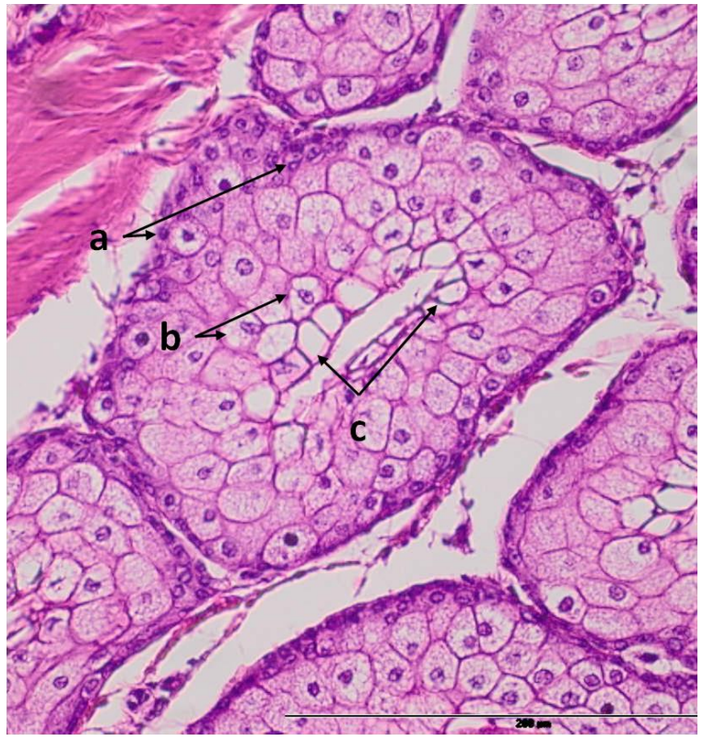


Figure 2.15: Photomicrograph of brown kiwi UG Midline section. Follicle cell types: a) germinative cells; b) intermediate cells; c) secretory cells. H&E scale bar = 200µm. Photograph by author.

Germinative cells were predominantly flattened, cuboidal, or a transition between the two (Figure 2.15). This layer was between 1-3 cells thick and consisted of cells with dense basophilic nuclei and cytoplasm. The intermediate layer was more variable and thickness ranged from 3-8 cells. These cells were most commonly polyhedral and contained numerous small vacuoles and centrally located nuclei. The secretory layer ranged from 1-3 cells thick and their cytoplasm did not stain, thus cells appeared to be empty, although lipids were probably removed during processing. Their nuclei were displaced to the side of the cell due to secretion accumulation. Cells in the degenerative layer were rarely seen as once apoptosis had occurred cellular debris was lost to the lumen (Figure 2.15).

2.3.3.1 Histomorphometrics

Mean follicular diameter was not significantly different between the midline and lateral sections of the UG (Paired t-test; t=0.267; df=9; p=0.796; n=10; Figure 2.16). There was no significant difference in mean follicular diameter when sex of the bird or species of the bird were compared (Table 2.2). Mean luminal diameter was not significantly different between the middle and outside sections of the UG (Paired t-test; t=0.180; df=9; p=0.861; n=10; Figure 2.17). Similarly there was no significant effect of sex or species of kiwi on mean luminal diameter (Table 2.2).

Table 2.2: Overview of statistical results of comparisons of sex (male vs. female) and species (brown kiwi vs. great spotted kiwi) on mean follicular diameter, mean luminal diameter, mean number cells in follicle epithelial layer, mean number germinative cells in follicle epithelial layer, mean number intermediate cells in follicle epithelial layer, mean number secretory cells in follicle epithelial layer. Bold values in 'p-value' column represent significant tests using a Bonferroni corrected p = 0.05/12 = 0.0042.

Variable		Statistical Test	Test Stat	N (total)	DF	P Value	Associated Figure
Follicle Diameter MIDLINE	Sex	Paired T-test	2.037	10	4	0.111	2.16
	Species	Mann-Whitney	-1.254	10		0.210	
Follicle Diameter LATERAL	Sex	Paired T-test	1.036	10	4	0.359	
	Species	Mann-Whitney	-0.114	10		0.909	
Luminal Diameter MIDLINE	Sex	Paired T-test	0.046	10	4	0.966	2.17
	Species	Mann-Whitney	-1.254	10		0.210	
Luminal Diameter LATERAL	Sex	Paired T-test	-1.019	10	4	0.366	
	Species	Mann-Whitney	-0.798	10		0.425	
Number of Cells MIDLINE	Sex	Paired T-test	5.154	10	4	**0.007**	2.18
	Species	Mann-Whitney	-0.570	10		0.569	
Number of Cells LATERAL	Sex	Paired T-test	2.697	10	4	**0.054**	
	Species	Mann-Whitney	-0.570	10		0.569	
Germinative Cells MIDLINE	Sex	Paired T-test	0.540	10	4	0.618	2.19
	Species	Mann-Whitney	-1.943	10		**0.052**	
Germinative Cells LATERAL	Sex	Paired T-test	1.709	10	4	0.163	
	Species	Mann-Whitney	-0.798	10		0.425	
Intermediate Cells MIDLINE	Sex	Paired T-test	2.149	10	4	0.098	2.20
	Species	Mann-Whitney	-2.058	10		**0.040**	
Intermediate Cells LATERAL	Sex	Paired T-test	0.143	10	4	0.894	
	Species	Mann-Whitney	-1.481	10		0.138	
Secretory Cells MIDLINE	Sex	Paired T-test	0.162	10	4	0.879	2.21
	Species	Mann-Whitney	-0.570	10		0.569	
Secretory Cells LATERAL	Sex	Paired T-test	0.484	10	4	0.654	
	Species	Mann-Whitney	-0.114	10		0.909	

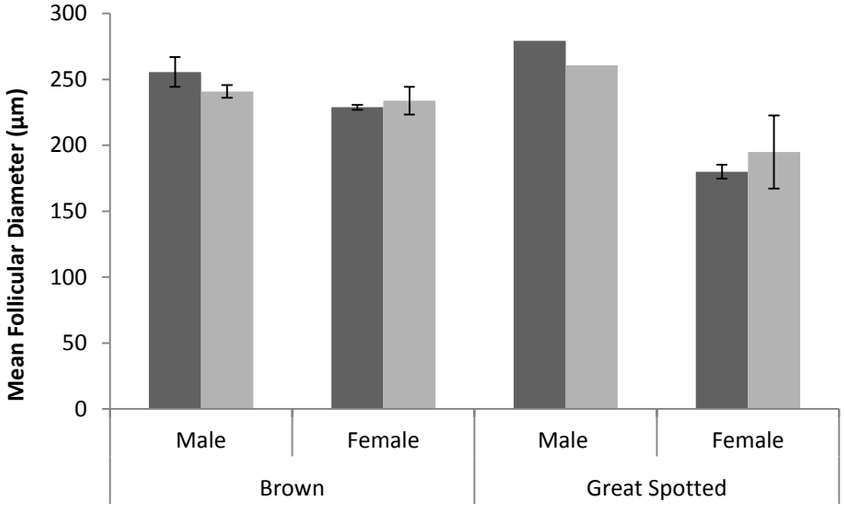


Figure 2.16: Comparison of mean follicular diameter of kiwi uropygial gland between sex and species (micrometers). Brown kiwi n= 4 males; 3 females; GSK n= 1 male; 2 females. Dark grey = midline section; light grey = lateral section. Error bars represent standard error.

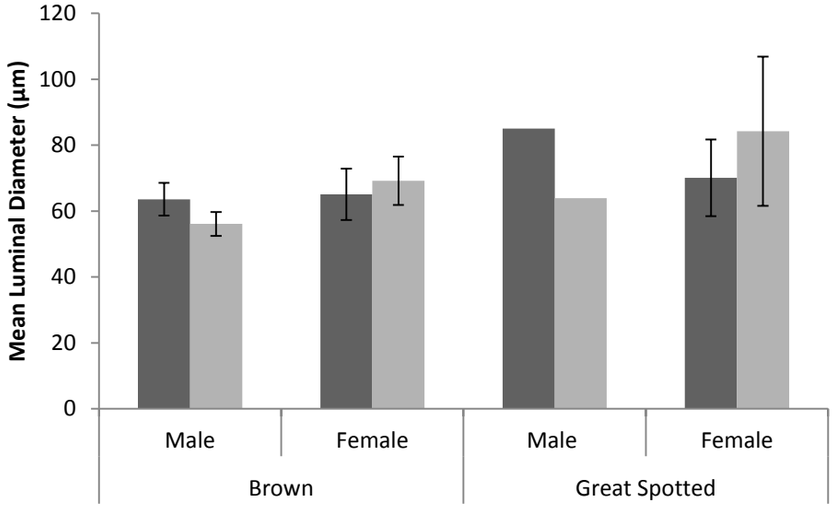


Figure 2.17: Comparison of mean luminal diameter of kiwi uropygial gland between sex and species (micrometers). Brown kiwi n= 4 males; 3 females; GSK n= 1 male; 2 females. Dark grey = midline section; light grey = lateral section. Error bars represent standard error.

The mean number of cells within the follicular epithelium was not significantly different between the middle and outside of the UG (Paired t-test; t=-0.654; df=9; p=0.529; n=10; Figure 2.18). On the other hand, females' UG contained significantly fewer cells in the follicular epithelium than males in the midline sections (Paired t-test; t=5.154; df=9 p=0.007; n=10; Figure 2.18) but not in the lateral sections (Paired t-test; t=2.697; df=9; p=0.054; n=10; Figure 2.18). When species of the bird was considered, however, there was no significant effect on the number of cells present in epithelial layers (Table 2.2).

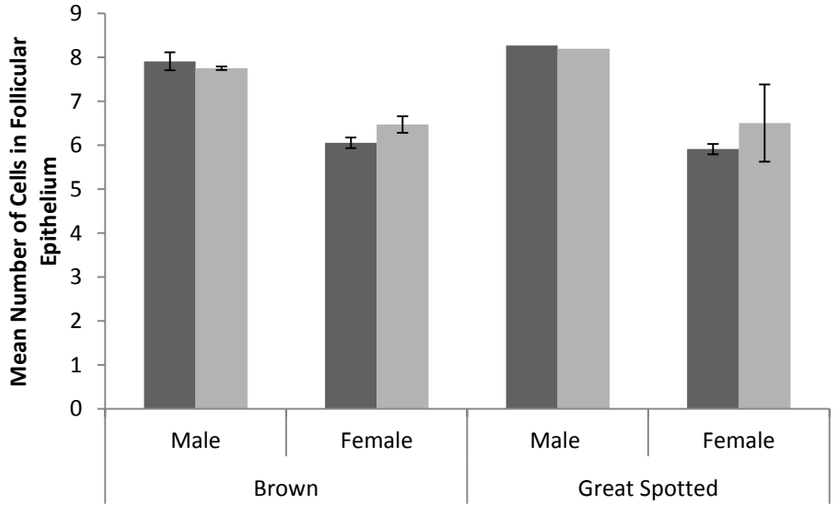


Figure 2.18: Comparison of mean number of cells counted in the follicle epithelium of kiwi uropygial glands between sex and species (micrometers). Brown kiwi n= 4 males; 3 females; GSK n= 1 male; 2 females. Dark grey = midline section; light grey = lateral section. Error bars represent standard error.

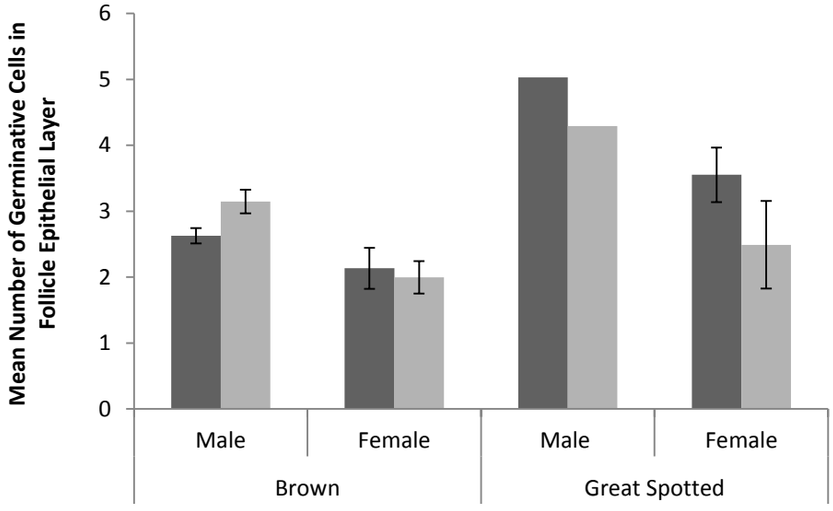


Figure 2.19: Comparison of mean number of germinative cells in the follicle epithelium of kiwi uropygial glands between sex and species (micrometers). Brown kiwi n= 4 males; 3 females; GSK n= 1 male; 2 females. Dark grey = midline section; light grey = lateral section. Error bars represent standard error.

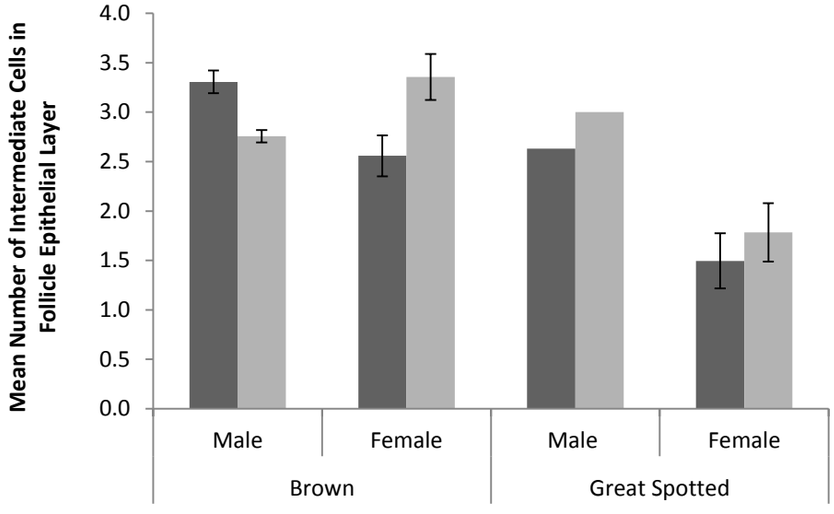


Figure 2.20: Comparison of mean number of intermediate cells in the follicle epithelium of kiwi uropygial glands between sex and species (micrometers). Brown kiwi n= 4 males; 3 females; GSK n= 1 male; 2 females. Dark grey = midline section; light grey = lateral section. Error bars represent standard error.

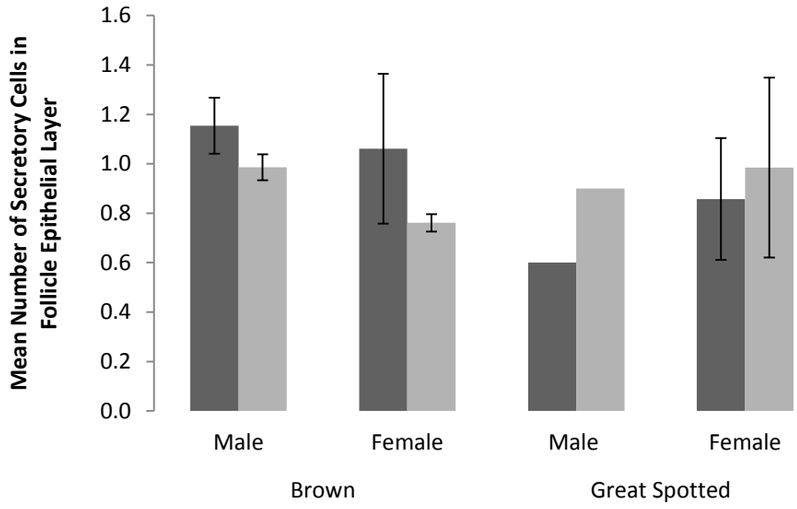


Figure 2.21: Comparison of mean number of secretory cells in the follicle epithelium of kiwi uropygial glands between sex and species (micrometers). Brown kiwi n= 4 males; 3 females; GSK n= 1 male; 2 females. Dark grey = midline section; light grey = lateral section. Error bars represent standard error.

The mean number of germinative cells in the follicular epithelium was not significantly different between the middle and outside of the gland (Paired t-test; t=0.467; df=9; p=0.651; n=10; Figure 2.19). There was no effect of sex on the mean number of germinative cells (Table 2.2), or species in the midline sections (Mann-Whitney; Z=-1.943; n=10; p=0.052; Figure 2.19). Similar to the germinative cell distribution, there was no significant difference in the number of intermediate cells encountered in the follicular epithelial layer between the middle and outside of the kiwi UG (Paired t-test; t=-0.520; df=9; p=0.620; n=10; Figure 2.20). In the midline section again there were no significant differences in any of the comparisons (Table 2.2). The mean number of secretory cells was not significantly different between the middle and outside of the kiwi UG (Paired t-test; t=0.605; df=9; p=0.560; n=10; Figure 2.21). No significant effects of either sex or species were found on the mean number of secretory cells (Table 2.2).

2.3.4 Pathology of the kiwi uropygial gland

The following four birds showed pathological changes in their UG's and were not utilised in the above histological examination.

2.3.4.1 Atrophy of the uropygial gland

Two cases of UG atrophy were observed, both occurring in sub-adult female great spotted kiwi. The first female presented with a history of progressive weight loss and at necropsy weighed 780g. She exhibited minimal epicardial fat reserves and had very little slightly reddened subcutaneous fat. The gastrointestinal tract was empty but no parasites (helminth or coccidia) were found, nor were any pathogenic bacteria cultured (e.g. *Salmonella* and *Campylobacter*). Histopathology revealed haemosiderin within liver Kupffer cells, as well as the presence of haemosiderin-laden macrophages in the parenchyma of the spleen. The UG showed severe generalised atrophy of epithelial elements (Figure 2.22 a & b). Starvation was diagnosed as cause of death in this bird.

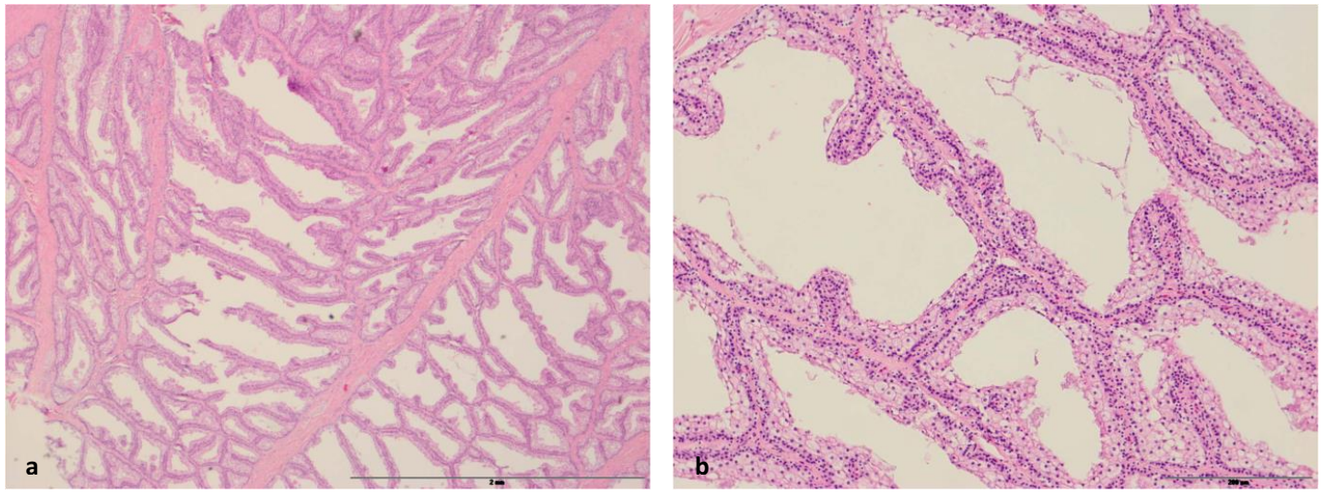
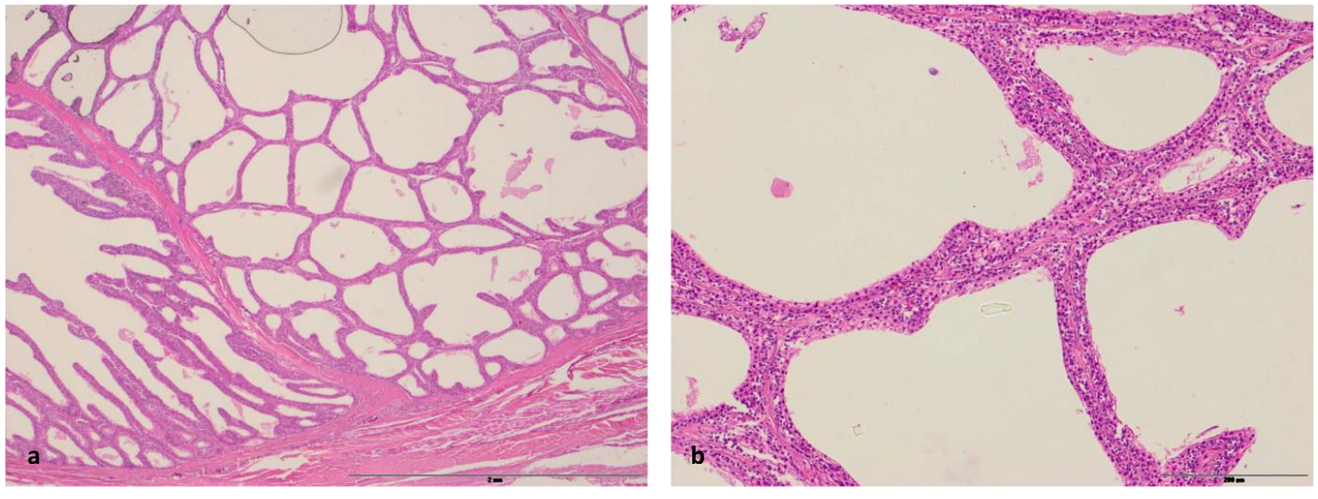


Figure 2.22: Photomicrographs of the UG in great spotted kiwi case one. a) Follicles are reduced in number and epithelial thickness is lacking; H&E scale bar = 2mm. b) Showing severely reduced epithelial thickness and expanded lumina of follicles; H&E scale bar =200µm. Photographs by author.

Case two was a great spotted kiwi from an urban native bush remnant in Christchurch that was recaptured after presenting with a positive faecal culture for *Yersinia*. During care there was difficulty in getting the bird to consume the captive diet, and after seven days she deteriorated, was very thin and died. On gross examination she had no epicardial or subcutaneous fat reserves and had severe generalised muscle atrophy. The lungs were very dark red in colour and contained 3-5mm yellow areas of necrosis. On histopathological investigation these areas of necrosis consisted of severe mycotic bronchopneumonia. Macrophages, lymphoid cells and multinucleate giant cells filled airways and air capillaries and surrounded a large number of fungal elements – later established as *Aspergillus*. The UG showed moderate generalised atrophy and the follicles throughout the gland were reduced in number and their lumina were diffusely expanded (Figure 2.23 a & b). The epithelium was markedly attenuated with no secretory cells present. The severity of atrophy of the UG in these two cases can be appreciated when a comparison is made with that seen in a normal great spotted kiwi of a similar age and sex (Figure 2.23 c).



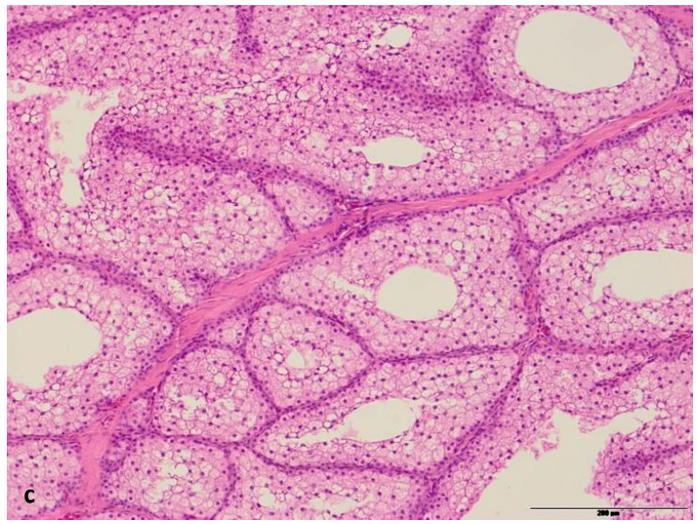


Figure 2.23: Photomicrograph of the UG in great spotted kiwi case two. a) Follicles are reduced in number and epithelial thickness is also reduced. No secretion is present within the follicles. H&E scale bar = 2mm. b) the epithelium is reduced to a single layer in many areas and the lumina are expanded H&E scale bar = 200µm. c) Photomicrograph of normal juvenile female great spotted kiwi UG gland. The follicle epithelium is thicker and follicle lumina are smaller in size than atrophied glands. H&E scale bar = 200µm. Photographs by author.

2.3.4.2 Pustular dermatitis of the uropygial gland

An adult female brown kiwi was found injured down the side of a bank close to a road. She was taken into care and underwent surgery to fix broken toes. Following this she was placed in a small outside run where she appeared to be doing well until she was found dead a week later.

Gross examination revealed that the bird was in good body condition with good internal fat reserves and musculature. There was a small amount of free blood in the abdomen and left thoracic cavity and a large clot of blood covered the ventral surface of the proventriculus and gizzard. The left lung was firm in texture and both presented with hyperaemia. As well as this the adrenal glands were enlarged bilaterally.

Histopathological investigation showed a severe diffuse bacterial ventriculitis which was thought to be the primary cause of death. The gizzard had completely lost the koilin layer and the underlying glands were distended with eosinophilic pyknotic debris, degenerated heterophils, and large numbers of bacteria streaming into the overlying lumen. The lamina propria, submucosa, muscle layers, and serosa all contained moderate numbers of heterophils. Bacteria present in the deep glands were Gram-negative, short rods, while bacteria within the superficial layers of remaining koilin were a mixture of Gram-negative, short rods and Gram-positive, medium sized rods. The lung showed an acute bronchopneumonia associated with aspirated koilin and bacteria. Occasional small parabronchial and pleural granulomas were also present.

An acute superficial dermatitis was present in the skin overlying the papilla of the UG. Superficial epithelial cells showed multifocal ballooning degeneration and heterophil infiltration of the stratum spinosum and the upper dermis was prominent. Between the stratum corneum pustule formation occurred in several areas extending into the stratum spinosum (Figures 2.24 & 2.25). In addition to the pustule formation, hyperkeratosis of the papilla also occurred (Figures 2.24 & 2.25). This type of pustular formation has been associated with avian pox virus in a variety of birds (Yoshikkawa & Alam, 2002).

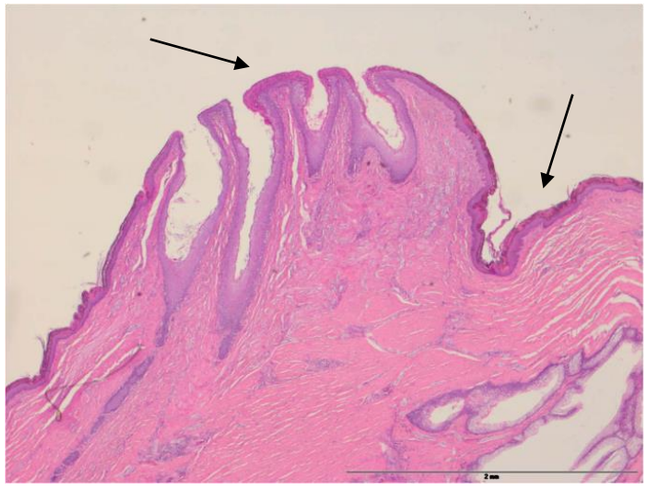


Figure 2.24: Photomicrograph of the uropygial papilla in a female brown kiwi showing hyperkeratosis and pustule formation (arrow). H&E scale bar = 2mm. Photograph by author.

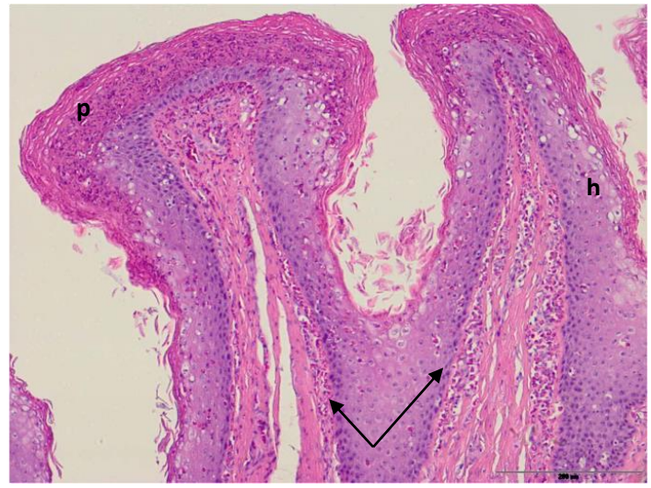


Figure 2.25: Photomicrograph showing hyperkeratosis (h) and pustule formation (p) of the papilla in a female brown kiwi. Arrows: heterophil infiltration of the dermis. H&E scale bar = 200µm. Photograph by author.

2.3.4.3 Uropygial gland adenitis

A sub-adult male Haast tokoeka was found dead under thick vegetation in a West Coast sanctuary. The carcass was in moderate to poor body condition with gelatinous reddening of the remaining subcutaneous fat reserves. On gross examination the coelomic cavity contained a large amount of dark yellow, turbid and flocculent fluid. Numerous thick plaques of yellow fibrinous material was adherent to most of the ventral body wall, the heart, left thoracic air sac, left liver lobe, the ventral aspect of the gizzard and proventriculus, and the serosal surfaces of the small and large intestines. The lungs were diffusely deep red, glistening and slightly swollen while no other abnormalities were discovered. The cause of death was a severe fibrinous coelomitis due to bacterial infection, possibly from an ingested foreign body which penetrated the wall of the gizzard where the infection was localised.

Histopathology of the UG revealed a localised adenitis (inflammation) in its ventral lobes. The ventral UG sinus was filled with necrotic keratinaceous debri (Figure 2.26) which contained numerous and widely distributed Gram-positive cocci (Figures 2.27). These bacteria were predominantly arranged in clumps although a few were scattered individually or in pairs (Figure 2.27). The surrounding glandular epithelium showed severe diffuse hyperkeratosis, squamous metaplasia, and hyperplasia (Figures 2.28). Some areas of the gland showed numerous epithelial pegs extending from the base of the UG epithelium into the surrounding connective tissue (Figure 2.29). At the base of the epithelium moderate numbers of infiltrating lymphocytes were present. Intraepithelial migrating granulocytes were also seen as well as a nodule of lymphocytes surrounding a capillary in the adjacent connective tissue.

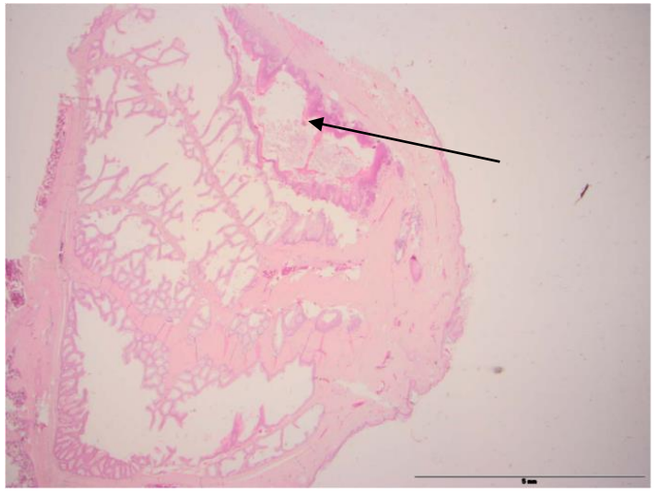


Figure 2.26: Photomicrograph showing Haast Tokoeka UG - ventral UG sinus filled with necrotic keratinaceous debri (arrow). H&E scale bar = 5mm. Photograph by author.

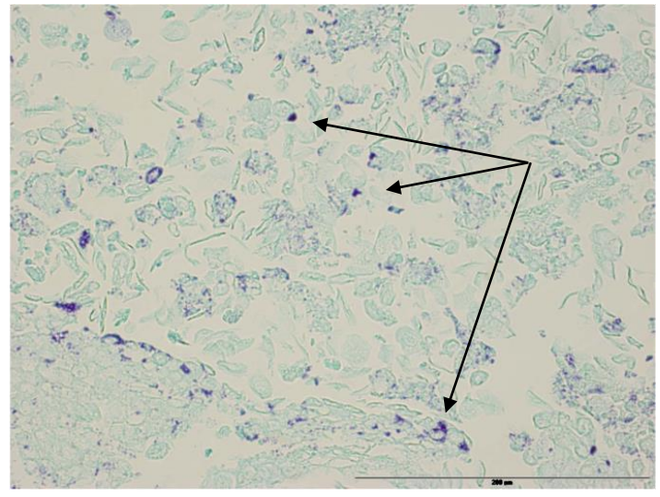


Figure 2.27: Photomicrograph showing Haast tokoeka UG - ventral UG sinus filled with necrotic keratinaceous debri and clumps/pairs/singles of Gram-positive cocci (arrows). Gram stain scale bar = 200µm. Photograph by author.

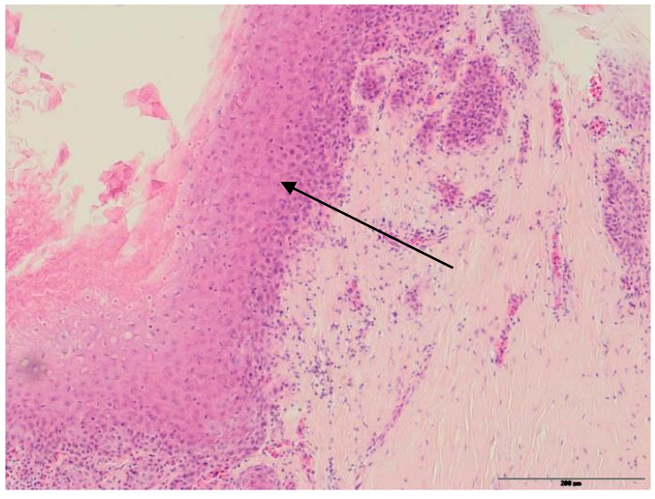


Figure 2.28: Photomicrograph showing severe diffuse hyperkeratosis, squamous metaplasia, and hyperplasia of affected glandular epithelium (arrow). H&E scale bar = 200µm. Photograph by author.

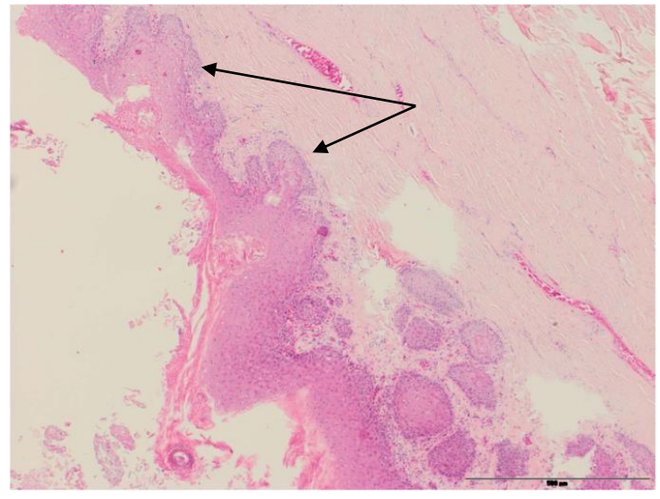


Figure 2.29: Photomicrograph showing numerous epithelial pegs extending from the base of the epithelium into the surrounding connective tissue (arrow). H&E scale bar = 500 µm. Photograph by author.

2.4 Discussion

This investigation has revealed that the UG of kiwi differs markedly from that of most other birds examined to date. Unlike most birds, the kiwi UG has eight primary sinuses, each of which is clearly associated with its own orifice, making eight openings in the papillae. The kiwi UG had no smooth muscle within the capsule, interlobular septae or interfollicular septae in contrast with most other species reported to date. It did, however, have striated muscle attached to the capsule whereby a tendon linked the two. Generally, in the 16% of birds studied to date, the UG has two lobes each with its own primary sinus and associated primary duct. Therefore, the 'standard' UG possesses two primary sinuses and two orifices (Jacob & Ziswiler, 1982), although there are exceptions to this rule. The hoopoe (*Upupa epops*) has three lobes which empty into a papilla that has one broad orifice (Jacob & Ziswiler, 1982; Martín-Vivaldi *et al.* 2009). Secretion pools within the papilla and is used in predator deterrence (Martín-Vivaldi *et al.* 2009). In contrast to this, the European nightjar (*Caprimulgus europaeus*) only has one lobe (Jacob & Ziswiler, 1982).

Jacob and Ziswiler (1982) discovered a proportion of species with more than two orifices (Table 2.3). In these species they found that the secondary sinuses led to the base of the papilla (no primary sinuses existed) where associated ducts continued through the papilla, opening at individual orifices. In the kiwi, however, storage sinuses associated with each duct running through the papilla were, in fact, primary sinuses, as these areas were large and had many secondary sinuses running into the parenchyma (Figure 2.12). Therefore, kiwi fall into the same group of birds studied by Jacob and Ziswiler (1982; Table 2.3) that possessed more than two orifices.

Table 2.3: Species found by Jacob and Ziswiler (1982) with more than two orifices plus findings from this study.

Species	Number of orifices	Family	Ecology
Fulmarus glacialis	8	Procellariidae	Seabird
Puffinus gravis	12	Procellariidae	Seabird
Diomedea exulans	8	Diomedeidae	Seabird
Phalacrocorax pygmaeus	16-20	Phalacrocoracidae	Seabird
Phalacrocorax carbo	16-26	Phalacrocoracidae	Seabird
Phalacrocorax aristotelis	12	Phalacrocoracidae	Seabird
Sula bassana	10-12	Sulidae	Seabird
Pelecanus onocrotalus	16	Pelecanidae	Seabird
Phoenicopterus ruber	8-10	Phoenicopteridae	Wetland bird
Phoeniconaias minor	6	Phoenicopteridae	Wetland bird
Ciconia ciconia	10	Ciconiidae	Wetland bird
Geronticus eremita	12	Threskiornithidae	Semi-arid bird
Grus grus	10	Gruidae	Forest/Swamp bird
Larus argentatus	8	Laridae	Seabird
Larus fuscus	8	Laridae	Seabird
Rissa tridactyla	8	Laridae	Seabird
Sterna paradisaea	8	Sternidae	Seabird
Sterna fuscata	6	Sternidae	Seabird
Sterna albifrons	8	Sternidae	Seabird
Sterna caspia	8	Sternidae	Seabird
Uria aalge	8	Alcidae	Seabird
Apteryx mantelli	8	Apterygidae	Ground insectivore
Apteryx australis	8	Apterygidae	Ground insectivore

It is interesting to note that most of the birds that have more than two orifices are birds affiliated with aquatic habitats (Table 2.3). Having many openings may allow the birds to obtain a larger amount of secretion from the gland while preening. A higher flow rate, however, would indicate a need for an increased amount of secretion synthesis and raises the possibility that birds with more than two orifices have differences in the cellular topography of their UGs. This could be investigated in the future, as well as the relation to aquatic life and/or wet environments (in the case of the kiwi).

The capsule, interlobular septa and interfollicular septae were all devoid of any smooth muscle fibres. This contrasts with species portrayed in Lucas and Stettenheim's (1972) and Jacob and Ziswiler's (1982) investigations, although it is similar to the situation in geese (Anatidae) (Hou, 1928), moorhen (*Gallinula chloropus*) (Sawad, 2006), and starling (*Sturnus vulgaris*) (Sadoon, 2011) UGs. The interfollicular septae in kiwi seem to be thicker in certain areas, causing the formation of lobules, a feature similar to the rock ptarmigan (*Lagopus mutus*) the Indian peafowl (*Pavo cristatus*) (Jacob & Ziswiler, 1982) and some reptiles (Weldon & Sampson, 1988). This contrasts with other species' UGs (e.g. water rail (*Rallus aquaticus*) and the great northern loon (*Gavia immer*)), where the interfollicular septae are thin and traverse the germinative cell layers of each individual follicle (Jacob & Ziswiler, 1982). Kiwi interfollicular septae are also different to the sebaceous glands of mammals, where no discrete follicle groupings occur (Schneider & Paus, 2010).

Studies of the internal and external musculature surrounding the UG are scarce. Lucas and Stettenheim (1972) and Jacob and Ziswiler (1982) provide brief descriptions, but are very general. Lucas and Stettenheim (1972) discovered the presence of smooth muscle within the papilla region of the chicken and stated that an important function of these transverse muscles is expansion of duct lumen. Thus, muscular contraction in the chicken causes the opening of the primary ducts and aids in secretion expulsion from the gland. The absence of internal smooth muscle fibres in the UG of kiwi and other species (Hou, 1928; Sawad, 2006; Sadoon, 2011), however, suggests that this is not the only way secretion is expelled from the UG and that another system must be in place. On the other hand, the presence of smooth muscle between the capsule and dermal layers of the integument of kiwi may be of some benefit in expelling secretion from the UG. Because smooth muscle was only present on the surface of the gland and never underneath, this remains uncertain.

Jacob and Ziswiler (1982) believe that the striated muscle of the tail region can have both direct and indirect effects on expulsion of secretion from the UG. In some species the *levator caudae* of the tail insert directly onto the

capsule of the UG, with contractions causing lateral and lift movements of the tail, and longitudinal extension of the UG (Jacob & Ziswiler, 1982). In the kiwi the presence of a tendon-like structure associated with underlying striated muscle and the capsule of the UG (Figure 2.11) may relate to these observations. Video recordings of kiwi on Ponui show that kiwi are able to control the spread of their tail feathers when defecating and preening and may be able to use these movements to squeeze out UG secretion. When obtaining secretion for experimental purposes the kiwi UG had to be warmed in order to allow the secretion to flow when it was massaged. Since kiwi were caught during the day, their metabolic rate would have been reduced and peripheral regions of the body much cooler than when the birds were active at night. Because kiwi have muscular control over the movement of their tail feathers it is likely that these movements aid in secretion expulsion during preening bouts when the gland is warm and secretion is able to flow smoothly.

Characteristics of the follicle cell layers in kiwi were similar to other species studied histologically, for example the osprey (*Pandion haliaetus*) (Harem *et al.* 2010), moorhen (Sawad, 2006), starling (Sadoon, 2011), and chicken (Lucas & Stettenheim, 1972). The thickness of each follicular epithelium was similar between individuals, although discrepancies arose within differing areas of an individual gland. These differences in follicle cell layers within the same gland may have been the result of inconsistencies when sectioning each UG. For example, sectioning each follicle through its midline is impossible because of its location within the UG itself – follicles are arranged complexly around secondary sinuses and are most commonly convoluted. This is similar to most other species studied histologically (Jacob & Ziswiler, 1982). The volume of the UG in kiwi was not affected by age or sex, but varied significantly between measurements (Pers. Obs.). Other factors which may affect the production of UG secretion are age, climate, diet, and season.

Male kiwi had significantly more cells contributing to their follicular epithelium than females. Therefore, male kiwi seemed to posses thicker follicular epithelia than female kiwi. This could have implications for the amount and/or

composition of the secretion produced. More cells may lead to an increased production of secretion as more cells would be liberated into the lumen of the follicle. There was no significant effect of species on the cellular topography of the UG secretory area. Only brown kiwi (*Apteryx mantelli*) and great spotted kiwi (*Apteryx haastii*) UGs were obtained for this analysis, so there could be an effect of species on the cellular characteristics of the UG when the other three species – *Apteryx owenii, Apteryx australis,* and *Apteryx rowi* – are taken into consideration. Size and age of the birds are variables that could not be controlled for in this investigation. Future studies could control for these variables to gain more insight into their effects.

Information gained from morphological investigations of UGs are pivotal to our understanding of the gland's function. The structural complexity of this gland and its existence among birds must indicate an importance of the secretion. Increased histological investigations may aid in explaining the variations seen among species and in turn may aid in unveiling chief functions for the secretion and its relationship to preening. It was interesting to find pathological changes in some of the birds sampled in this investigation. Systemic illnesses seem to affect the UG and this in turn has implications on the overall condition of the bird – both physically and ecologically. Avian pox has recently been reported in brown kiwi (Ha *et al.* 2011); and the discovery of pustular dermatitis of the UG in an adult female brown kiwi may be an early case of this disease.

Both the control system for the expulsion of secretion and the innovation of secretion synthesis require further investigation in kiwi. Few studies have examined the role of hormones in the control of secretion production. In the pigeon treatment with the hormone estriol resulted in inhibition of UG activity (Manna *et al.* 1983) while in mallards, estradiol increased the number of peroxisomes (associated with fat biosynthesis (Sara *et al.* 2006)) within secretory cells and consequently enhanced the fatty acid diester biosynthesis (Bohnet *et al.* 1991). Furthermore, hormones control the synthesis of secretion in sebaceous glands of mammals (Schneider & Paus, 2010). This aspect

of control warrants further investigation in the life history of birds as hormones could unveil key information regarding the gland's function.

Future studies should move toward investigating the histological organisation and structure of the UGs of Tinamiformes, as this would complete the information known about UG's of the palaeognathae lineage. It would be interesting to see whether there are differences between the UG of tinamous species or whether each species' UG has a similar morphology. Due to the lack of UGs in the adult of any other ratites, environmental pressures placed on kiwi and tinamous may have caused the retention of the UG in these birds. Species in these two orders are small and are most commonly ground dwellers – kiwi nest and roost in the ground. The lack of the UG in adults of the other ratite species – which are much taller – gives a hint that the UG in kiwi and tinamous may provide benefits to their ground dwelling existence. Histological comparison of the UG in these two orders will aid in answering these questions and will also assist in closing the knowledge gap on this still enigmatic gland.

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Chapter 3: The Comparative Morphology of the Uropygial Gland in Honey-eating Forest Birds of New Zealand.

ABSTRACT

Both molecular techniques and morphological characters have been important in determining the taxonomic position of the endemic honeyeaters of New Zealand. New Zealand has two Meliphagidae species, the tui (*Prosthemadera novaeseelandiae*) and the New Zealand bellbird (*Anthornis melanura*). The hihi (stitchbird) (*Notiomystis cincta*) was once classified within the Meliphagidae, but genetic analysis has recently separated hihi into its own monotypic family – Notiomystidae. Its closest relatives are thought to be the saddleback (*Philesturnus carunculatus*) and the kokako (*Callaeas cinirea*), two New Zealand wattlebirds in the family Callaeidae. The current study has used the morphology of the uropygial gland as a means of comparing the taxonomic status of these endemic species. The results support the taxonomical position of tui, bellbirds, hihi, and saddleback within the order Passeriformes. Each of their UG's possessed features distinct to this order, in that they had two lobes with a 'wart-like' papilla. Bellbirds possessed the largest gland of the four species. When tui, bellbirds, hihi, and saddleback are compared to passerine species examined by Jacob and Ziswiler (1982), they grouped closely together. These results support the hypothesis of an ancient radiation of the New Zealand passerines from a common ancestor in New Zealand when it first split from Gondwana.

3.1 Introduction

New Zealand's endemic avifauna diverges extensively in many life history traits when compared with relatives in other areas of the world (Driskell *et al.* 2007). For this reason, uncertainty surrounds the evolutionary relationships of many New Zealand avian species, which may impact on the overall recognised biodiversity of the country. Upon human colonisation of New Zealand, introduced predators, disease, and the reduction of native forests by 70% on the mainland, destroyed the once thriving avifauna of New Zealand (MacPhee, 1999; Ewers *et al.* 2006). This has resulted in a depauperate representation of the country's original avian biodiversity, with over 40 avian extinctions (MacPhee, 1999). Many New Zealand birds are now classified by the Department of Conservation as 'at risk' or 'threatened' (Hitchmough, 2012). Rigorous conservation of these endangered species has been an essential part of maintaining New Zealand as one of the biodiversity hotspots of the world (Driskell *et al.* 2007).

New Zealand has two endemic Meliphagidae (honeyeater) species – the tui (*Prosthemadera novaeseelandiae*) and the New Zealand bellbird (*Anthornis melanura*). The hihi (*Notiomystis cincta*) was once classified within the Meliphagidae but recent genetic research has placed the species in its own monotypic family, the Notiomystidae (Driskell, 2001; Driskell *et al.* 2007). Members of Callaeidae (e.g. saddleback – *Philesturnus carunculatus*) are now thought to be hihi's closest relatives (Ewen *et al.* 2006; Driskell *et al.* 2007), with a divergence time between the two groups of approximately 28-39mya (Driskell *et al.* 2007).

Similar to many other New Zealand birds, today's bellbird abundance does not represent original numbers (Craig & Douglas, 1984). Instead, the distribution of this species over the country reflects the remaining area of native forest (Ewers *et al.* 2006; Baillie, 2011) – their preferred habitat (Higgins, Peter, & Steele, 2001). As with the bellbird, the tui has also succumbed to the pressures of human colonisation (Diamond & Veitch, 1981) and although it remains a relatively common feature in New Zealand gardens, its population is generally declining (Elliott *et al.* 2010). The hihi is one of the most endangered birds in New Zealand. This species was originally

distributed throughout the North Island mainland, Great Barrier Island, Hauturu (Little Barrier Island), and Kapiti Island. However, already *i*n 1873 hihi populations were reducing in numbers and by the 1880's populations of hihi had declined to a single surviving population on Hauturu (Taylor, Castro & Griffiths, 2005).

Unlike members of the Meliphagidae, hihi lacks the classic honeyeaters' canulated tongue, and the multiple foramina on the dorsal face of the sternum's midline (Driskell et al. 2007). Both molecular techniques and morphological features have, therefore, been important in discerning the relationships between groups of endemic New Zealand passerines. Within the Passeriformes Jacob and Ziswiler (1982) believe that the UG has "significant diagnostic value" for phylogenetic purposes. The recent alteration in the taxonomy of hihi provides an opportunity to compare its UG with other New Zealand passerines – in particular bellbirds, tui and saddleback – in order to investigate the similarities/differences between them and thus offer support (or not) to their taxonomic status.

3.2 Materials and methods

3.2.1 Study Species

Dead birds preserved in 10% neutral buffered formalin were sourced from the archived collection at Massey University's Wildlife Health Centre. Ten hihi, three New Zealand bellbirds, three tui, and three saddleback were used in the investigation. These four species were selected because of their taxonomical status within the New Zealand passerine lineage. Hihi and the New Zealand bellbird are similar in size, but male tui are on average three times their size and male saddlebacks twice their size (Table 3.1; Heather & Robertson, 1996). These differences should be considered below in relation to the UG.

Table 3.1: Average male and female weights (grams) for the four study species. Tui is the largest, followed by the saddleback, hihi and bellbird. (Heather & Robertson, 1996).

	Hihi	Bellbird	Tui	Saddleback
Male	40g	34g	120g	80g
Female	30g	26g	90g	70g

3.2.2 Morphological examination

UGs were dissected from the carcasses with a margin of tissue to include the surrounding fat. This decreased the chance of accidentally slicing the gland in the wrong orientation. Measurements of the external anatomical features of each UG were made including length, width, and depth (Figure 3.1a). The tarsus length of each bird was also recorded.

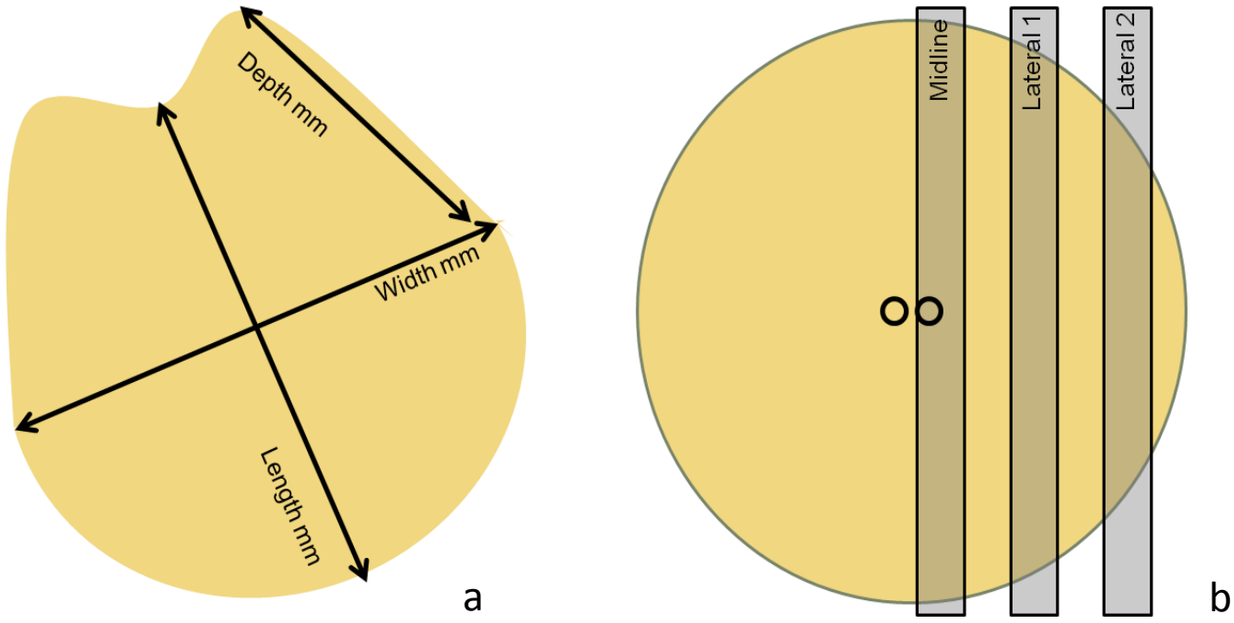


Figure 3.1 a) A model illustrating morphological features measured: length, width, and depth; b) A model illustrating histological sections produced from each UG - the two small circles in the middle represent dorsal view of papilla. Illustrations by author.

3.2.3 Histological examination

Each gland was sectioned sagittally into three slices. Initially, a section was made down the midline of the gland between the two papillae (Figure 3.1b). One side of the gland was then used to produce three sections – a midline section, and two lateral sections (lateral 1 and 2).

Each slice was embedded in Leica Histo Embedder (Leica Paraplast® Tissue Embedding Medium (Melting Point 56ºC)) and sectioned to 4 micrometres in size using a Leica RM2235 Manual Rotary Microtome with a S35 Feather Microtome Blade (stainless steel). Sections containing bone were decalcified before being cut to avoid tearing the glandular region of tissue. Decalcification (the process of inorganic calcium removal) consisted of sections being soaked in osteomoll (Merck) overnight prior to processing. Each tissue section on the slide (Menzel-Glaser Superfrost PLUS Slides 76 x 26mm Ground edge 90º) was then routinely stained with Haematoxylin and Eosin (H&E) and was coverslipped wet via a xylene bath (Leica CV 5030 robotic coverslipper; Coverslips = Menzel-Glaser 22 x 50 No. 1; Mountant = Entellan ® rapid mounting medium for microscopy). To investigate the structure of the hihi UG a Massons Trichome stain was used to identify connective tissue and muscle. Techniques followed procedures in Bancroft and Gamble (2008).

3.2.3.1 Histomorphometrics

Using photomicrography and image analysis software (Colourview digital camera and an AnalySIS 5 soft-imaging analysis system) a longitudinal transect of each section was made representing the maximum glandular diameter at 4x magnification (Figure 3.2). The number of follicles along this transect were counted in order to obtain a measure of follicular density. The mean follicular diameter and mean luminal diameter of each follicle along the transect were measured at 40x magnification. The number of cells contributing to the follicle cell layer and their degree of vacuolation (see section 2.3.3) were then recorded.

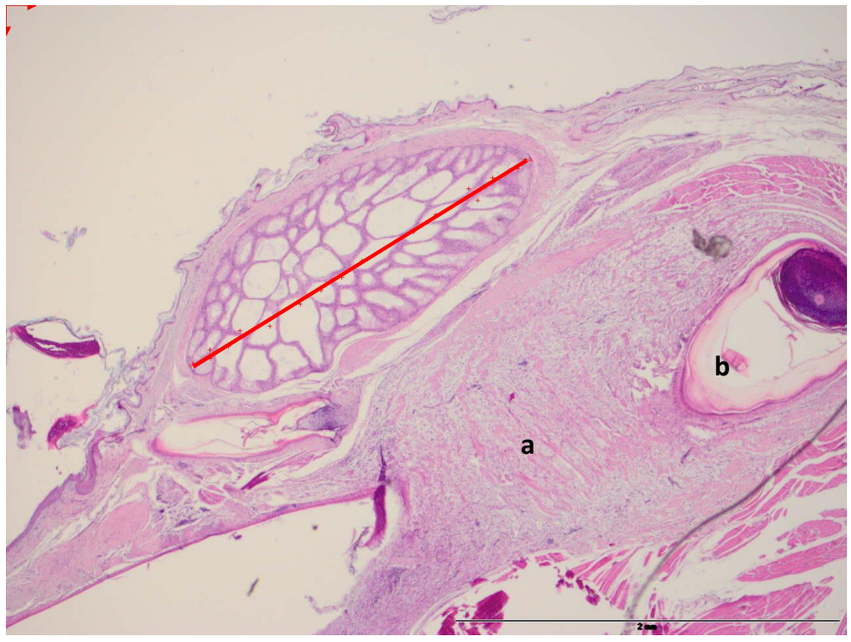


Figure 3.2: Photomicrograph of the UG of a New Zealand bellbird showing the longitudinal transect of maximum glandular diameter (red line). a) coccygeal bone of the tail, b) feather follicle. H&E; scale bar = 2mm. Photomicrograph by author.

3.2.4 Statistical analysis

Normality tests were carried out on all data and only the external characteristics were normally distributed. Therefore, to compare the external characteristics of the UG between species the mean and standard error was used. To compare follicle characteristics (which were not normally distributed) between species Kruskal-Wallis tests were employed.

All statistics are given to three decimal places. A Bonferroni corrected p value of 0.0042 was used. The reason for this is that when many tests are performed on a set of data, the null hypothesis is more likely to be rejected when it is true ("Type I" error) than when it is false. One strategy to guard against making a mistake is to make the alpha level more stringent. This can be achieved either by calculating the Bonferroni corrected p value, or 0.05/number of tests performed.

3.3 Results

3.3.1 Gross morphology of the uropygial gland

3.3.1.1 Hihi

The UG of hihi was bipartite in nature (Figure 3.6). The papilla was distinctly separated from the lobes (Figure 3.6) and had a mean length of 1.31mm, a mean width of 1.63mm and a mean depth of 0.61mm (Figure 3.4; Table 3.2). Hihi UGs lacked feathers on the lobes or papilla (Figures 3.8A). The lobes had a mean length of 8.62mm, a mean width of 5.43mm and a mean depth of 3.79mm (Figure 3.3; Table 3.2). A rim of connective tissue encapsulated the UG. This capsule was thickest at the most dorsal aspect of the gland, while ventrally it was much thinner (Figure 3.8a). The UG capsule of the hihi contained no melanin pigment. The gland was situated immediately dorsal to the tail feather follicles (Figure 3.8a) and no muscle was directly associated with it. At the periphery of the gland interfollicular septae branched into the gland from the capsule (Figure 3.5). These septae were thin and surrounded every follicle (Figures 3.5 & 3.8a). The septae merged in the middle of the gland to form thick bands of collagen and elastic fibres (Figure 3.8a).

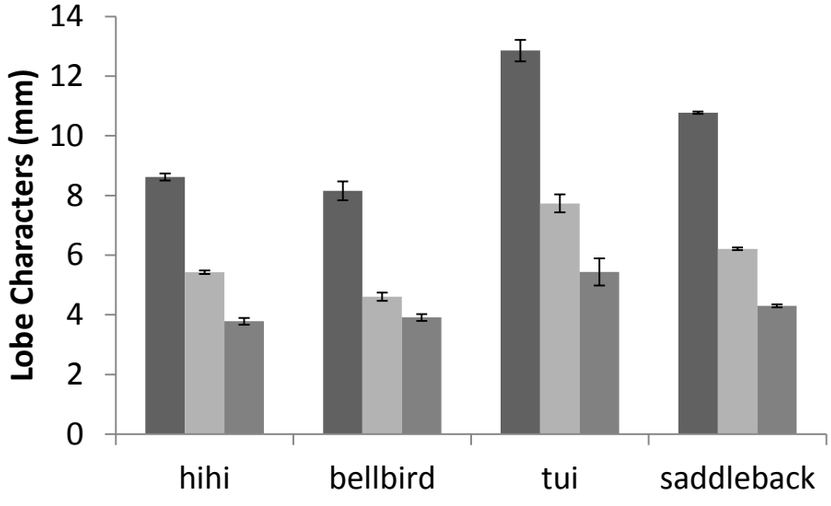


Figure 3.3: Lobe characteristics for each species. Dark grey = mean lobe length; light grey = mean lobe width; medium grey = mean lobe depth. Error bars represent standard error.

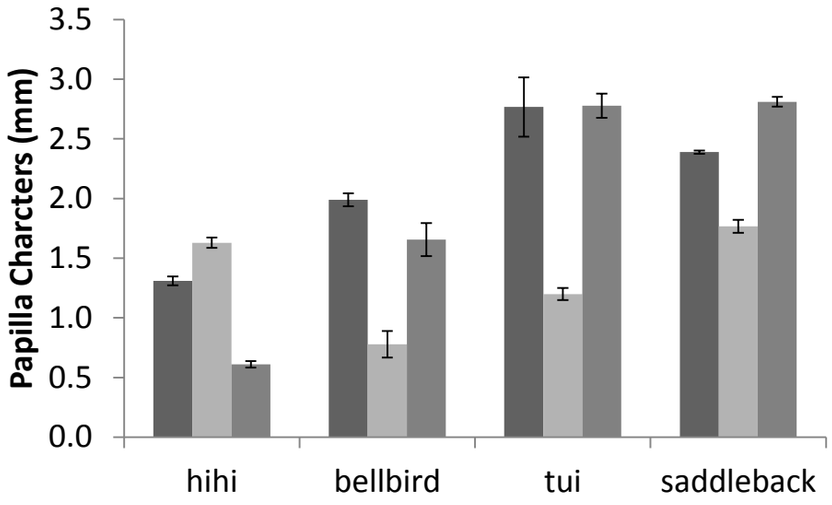


Figure 3.4: Papilla characteristics for each species. Dark grey = mean papilla length; light grey = mean papilla width; medium grey = mean papilla depth. Error bars represent standard error.

Table 3.2: Mean and standard deviation for external lobe and papilla characters: lobe length, lobe width, lobe depth, papilla length, papilla width, and papilla depth.

Variable	Hihi	Bellbird	Tui	Saddleback
Mean Lobe Length	8.62	8.16	12.86	10.78
Standard Error Lobe Length	0.12	0.32	0.36	0.04
Mean Lobe Width	5.43	4.61	7.73	6.21
Standard Error Lobe Width	0.06	0.14	0.30	0.04
Mean Lobe Depth	3.79	3.91	5.43	4.30
Standard Error Lobe Depth	0.11	0.11	0.45	0.05
Mean Papilla Length	1.31	1.99	2.77	2.39
Standard Error Papilla Length	0.04	0.05	0.25	0.01
Mean Papilla Width	1.63	0.78	1.20	1.77
Standard Error Papilla Width	0.04	0.11	0.05	0.06
Mean Papilla Depth	0.61	1.66	2.78	2.81
Standard Error Papilla Depth	0.03	0.14	0.10	0.04

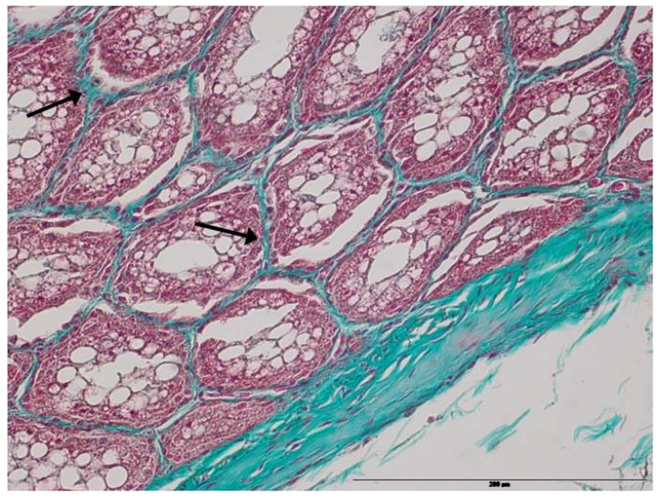


Figure 3.5: Photomicrograph of hihi UG showing thin interfollicular septae (black arrows). Massons Trichome stains connective tissue blue; scale bar =200µm. Photograph by author.

The isthmus of the papilla separating the two primary ducts was thin (Figure 3.6). The hihi papilla resembled the common passeriform type described by Jacob and Ziswiler (1982) as "the wart-like papilla of the Passeriformes". There were three primary sinuses in each lobe (Figure 3.7) and there was a valve composed of elastic and collagen fibres which formed a thick connective tissue band attached to the side of the papilla (Figure 3.8a). The capsule around the papilla was thick at its base and thinned towards the orifice (Figure 3.8a). There were only two orifices in the papilla, each associated with its own primary duct. The integument covering the papilla was composed of a layer of keratinised stratified squamous epithelial cells (Figure 3.8a).

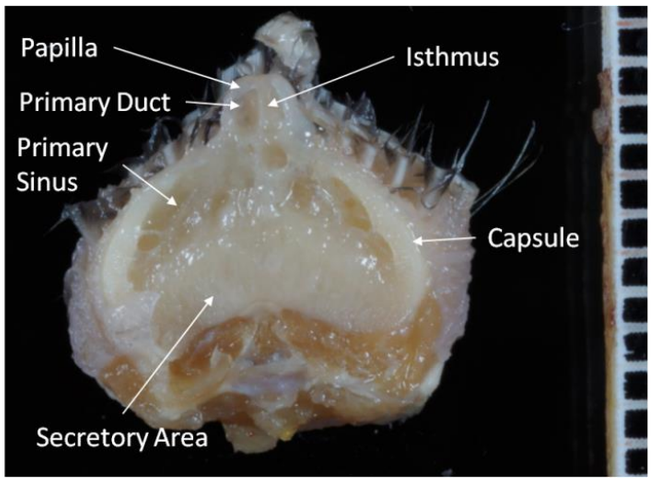


Figure 3.6: Hihi UG anatomical organisation. Mid transverse section. Scale graduations= millimetres. Photograph by author.

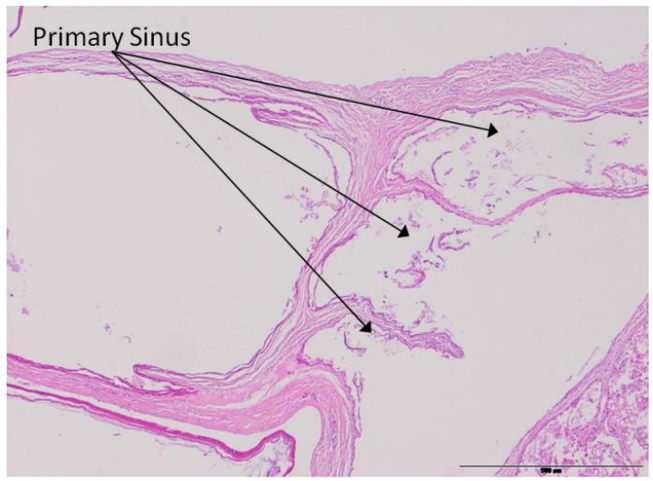


Figure 3.7: Photomicrograph of hihi UG showing three distinct primary sinuses. H&E; scale bar = 500µm. Photograph by author.

3.3.1.2 Bellbird

Like the hihi, the bellbird UG was bipartite in nature and there was a clear division between the lobes and papilla (Figure 3.8B). There was no feathering on the papilla or the lobes (Figure 3.8B). The average length of the lobes was longer than the width of the lobes which in turn was longer than their depth (Figure 3.3; Table 3.2). The papilla, however, had a longer depth than width (Figure 3.4; Table 3.2). The capsule of the bellbird UG was thin ventrally and thickened dramatically at the dorsal end (Figure 3.8b). The papilla of the bellbird UG was unusual in that it was formed from a dense array of connective tissue and did not conform to the typical 'wartlike papilla of the Passeriformes' (Figure 3.8b). Because of this it was difficult to observe a valve system typical of other passerine species within the papilla, although it was visible in some sections. The gland also clearly showed four primary sinuses within each lobe, thus expressing eight in total. Interfollicular septae did not seem to merge in the middle of the lobe and were thin (Figure 3.8b).

An isthmus was difficult to identify due to the denseness of the papilla connective tissue, as were the number of orifices associated with the papilla. As in other passerines the bellbird UG sat above the tail feathers (Figures 3.8B

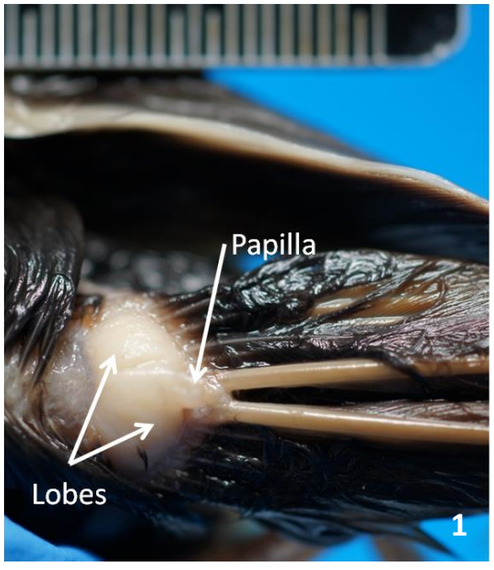
& 3.8b) thus feather movement may enable expulsion of the secretion as no skeletal muscle fibres were directly associated with the gland. The integument covering the papilla showed keratinisation at the most dorsal point.

3.3.1.3 Tui

The UG of tui were bipartite, naked (Figure 3.8C), and represented the typical passerine form with a distinct papilla separated from the lobes (Figure 3.8C). The average length of the lobes was 12.86mm, the average width was 7.73mm, and the average depth was 5.43mm, the largest of all four species examined (Figure 3.3; Table 3.2). The papilla was similar in size to that of the saddleback (Figure 3.4; Table 3.2). The capsule was thin ventrally and slightly thickened towards the papilla. Some merging of the interfollicular septae occurred in the middle of the lobe (Figure 3.8c). Three clear primary sinuses were apparent in the tui UG and these led into a typical 'wart-like papilla of the Passeriformes' (Figure 3.8c). The valve system was clearly present within the papilla to control the release of secretion (Figure 3.8c). Again the UG was positioned above the tail feathers (Figure 3.8c) and had no muscle directly associated with it.

3.3.1.4 Saddleback

The saddleback UG was bipartite and naked and it had a prominent papilla (Figure 3.8D), with the depth of the papilla being larger than its length (Figure 3.4; Table 3.2). Like the other passerines however, the papilla and lobes still expressed a clear division (Figure 3.8D). The ventral capsule was thin and thickened slightly towards the papilla (Figure 3.8d). There was no merging of interfollicular septae in the middle of the gland and follicles were arranged more as tubules around the primary sinuses than as semi-spherical acini (Figure 3.8d). There were three primary sinuses and these led to a typical 'wart-like papilla of the Passeriformes' (Figure 3.8d). There was a clear valve system to control the expulsion of the secretion. Unlike the other three species in this study, the saddleback UG did not sit directly over the tail feathers, instead it was positioned over the coccygeal bone of the tail (Figure 3.8d).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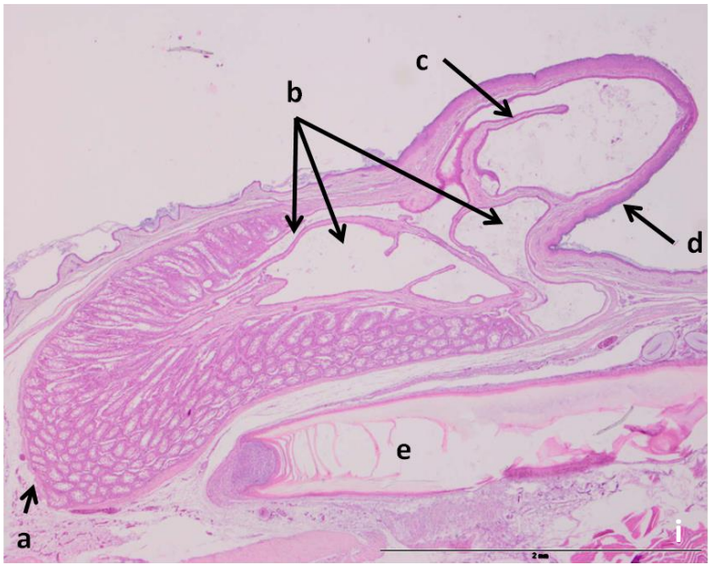
Lobes
1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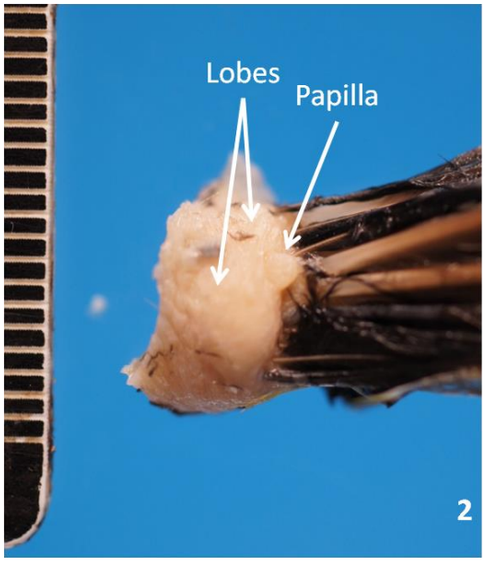
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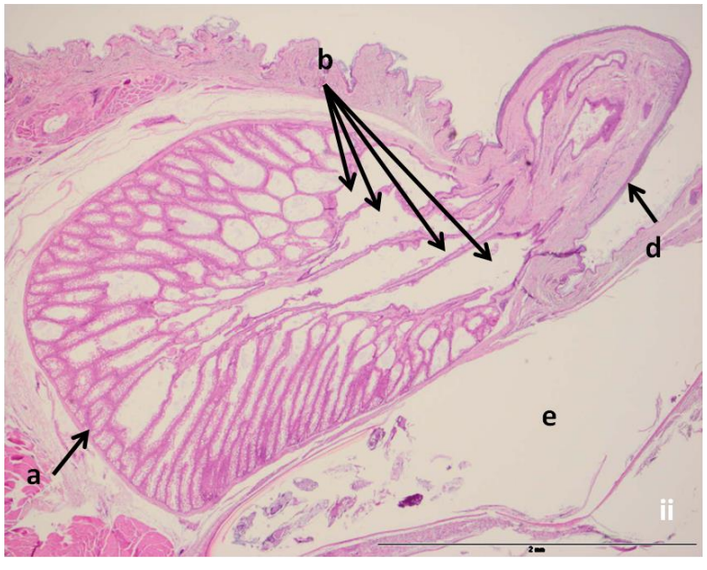
2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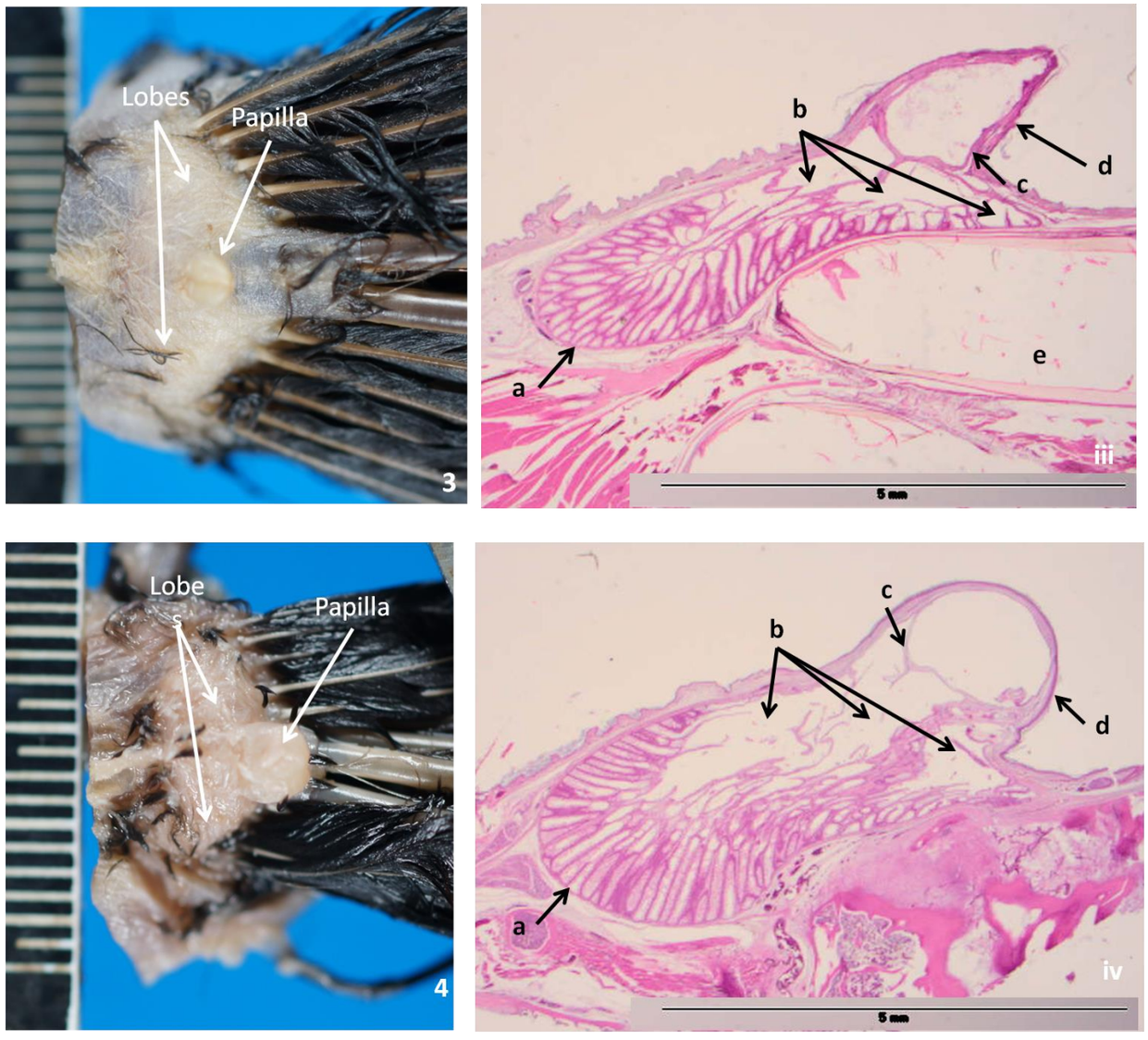

Figure 3.8: 1,2,3, & 4: Photographs of ventral view of UG showing the bipartite nature and nakedness of the lobes and papilla. Scale = millimetres. i, ii, iii, & iv: Photomicrographs of UG showing a) thin ventral capsule; b) primary sinuses; c) papilla valve; d)papilla; e) tail feather follicle. 1.i = hihi; 2.ii = New Zealand bellbird; 3.iii = tui; 4.iv = saddleback. H&E i & ii (scale bar = 2mm), iii & iv (scale bar = 5mm).

3.3.2 Uropygial gland follicular characteristics

The cell types in the UG of each species were classified into three zones as they all appeared similar: 'germinative', 'intermediate', and 'secretory' (Figure 3.9) (Lucas & Stettenheim, 1972; Jacob & Ziswiler, 1982). The germinative layer of cells consisted of those that were densely stained with a uniform, eosinophilic cytoplasm indicating that

very little secretion had accumulated (Figure 3.9; Jenik, Fisch, & Goodridge, 1987). The intermediate layers had cells with small vacuoles of secretion within their cytoplasm (Jenik, Fisch, & Goodridge, 1987) and were distinguished from the secretory cells by their centrally located nuclei (Figure 3.9). In secretory cells all the cytoplasm was filled with secretory product as the small vacuoles of the intermediate cells coalesced into one large vacuole (Figure 3.9). In these cells nuclei were displaced peripherally and they corresponded to the secretory and degenerative areas described by Jacob and Ziswiler (1982). The degenerative cells were much flatter, smaller and more disrupted than the underlying secretory cells (Jenik, Fisch, & Goodridge, 1987).

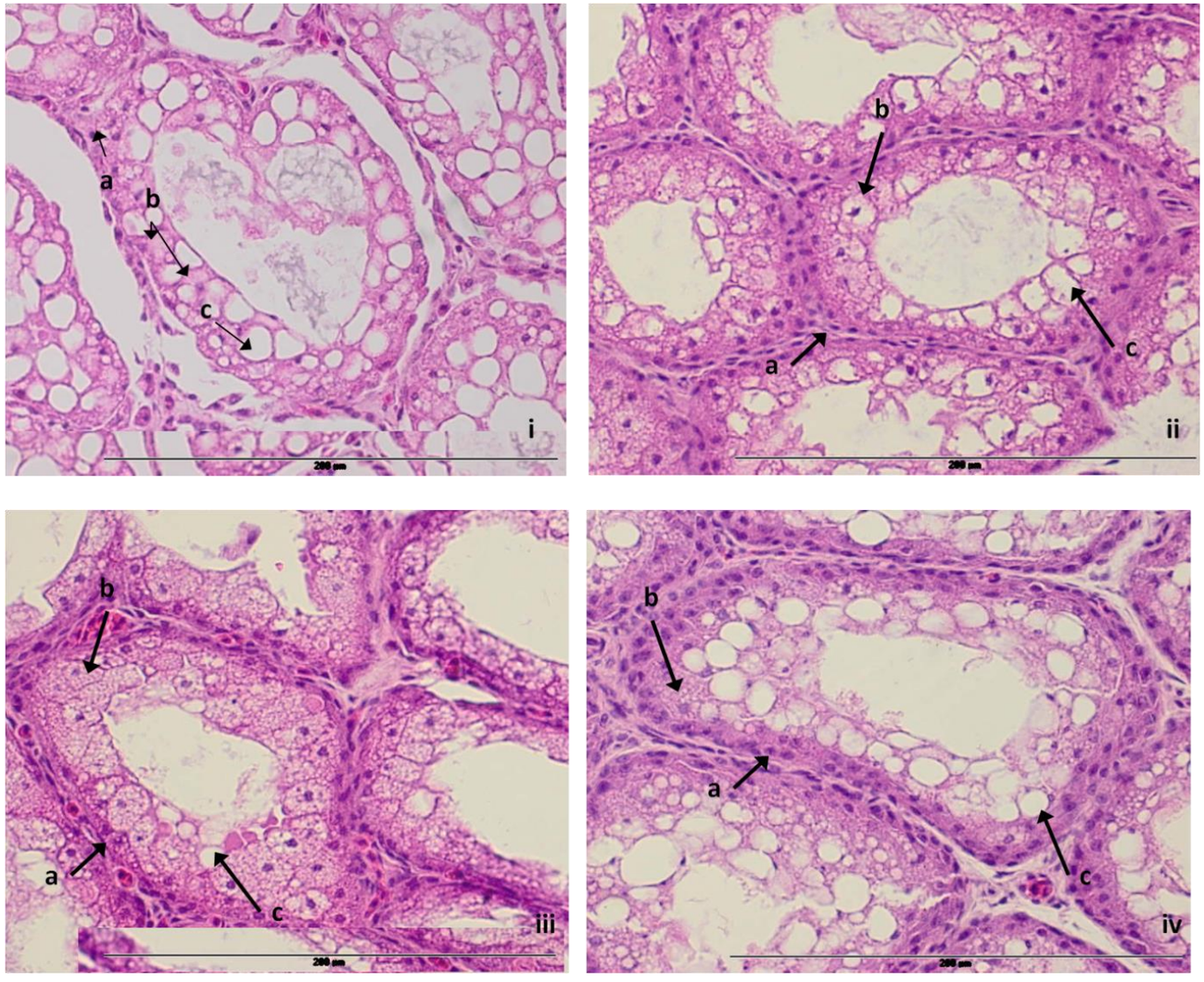

Figure 3.9: Photomicrograph of UG follicle cell types: a) germinative cells; b) intermediate cells; c) secretory cells. i = hihi; ii = New Zealand bellbird; iii = tui; iv = saddleback. H&E; scale bar = 200µm. Photograph by author.

3.3.2.1 Hihi

The germinative cells of the hihi follicle were thin, flattened in shape and were most commonly one cell thick (Figure 3.9i). The intermediate layer was scanty and thickness ranged from 1-3 cells. These cells were most commonly polyhedral with the cytoplasm of some showing a spongy appearance due to the presence numerous small vacuoles (Figure 3.9i). The secretory layer ranged from 1-3 cells thick and the cytoplasm did not stain, suggesting that they were full with secretory products. Nuclei and other cellular organelles were displaced to the side of the cell due to the accumulated secretion. This layer predominated in the hihi follicles and was not restricted to the central superficial area within it and vacuolated cells were often found near the base. Cells in the degenerative layer were seldom seen as once apoptosis had occurred cellular debris was lost into the lumen.

3.3.2.2 New Zealand bellbird

The bellbird UG follicle had prominent cell layers that were easily identified in comparison to the hihi follicles. The germinative cells ranged from flattened to cuboidal in shape and were in 1-2 cell layers thick (Figure 3.9ii). Intermediate cells in the bellbird were the dominant cell types seen in the follicles (Figure 3.9ii). This layer ranged from 1-7 cells in thickness and cells were commonly polyhedral in shape. The secretory layer of the bellbird follicle was generally absent, although, when present, ranged from 1-5 cells in thickness. Like the hihi, cells in the degenerative layer were not seen.

3.3.2.3 Tui

The tui UG had a definite lack of secretory cells in the secretory layer of the follicle. Because of this, the intermediate cell layer predominated, with the germinative layer consisting of only 1-2 flattened cells (Figure 3.9iii). Like the hihi and bellbird, cells in the degenerative layer were not seen.

3.3.2.4 Saddleback

The saddleback follicular structure was different from the other three species (Figure 3.9iv). The germinative cell layer consisted of 1-3 cuboidal cells and was a prominent layer in many saddleback follicles (Figure 3.9iv). The intermediate layer was small in this species and at most only consisted of four cells. The secretory cells were the predominant cell type in the saddleback follicle (Figure 3.9iv). The secretory layer consisted of up to five layers of cells in some follicles.

3.3.3 Histomorphometrics

Follicle diameter was significantly different in all four species in both the midline (Kruskal-Wallis; *H*=40.139; df=3; p=0.000; Figure 3.10a; Table 3.3) and lateral sections (Kruskal-Wallis; *H*=158.427; df=3; p=0.000; Figure 3.10b; Table 3.3), with hihi possessing the smallest diameter (Figure 3.10). Similarly, there was a significant difference in lumen diameter between species, both in the midline (Kruskal-Wallis; *H*=142.356; df=3; p=0.000; Figure 3.11a; Table 3.3) and laterally (Kruskal-Wallis; *H*=176.880; df=3; p=0.000; Figure 3.11b; Table 3.3) and hihi had the smallest follicular lumina of all four species (Figure 3.11).

Table 3.3: Descriptive statistics for UG follicular characteristics of hihi, bellbirds, tui, and saddleback. N = number of follicles examined.

Variable	Statistics	Hihi	Bellbird	Tui	Saddleback
Midline Follicle Diameter	Median	116.3	155.4	146.9	146.5
	Range	424	306	282.9	248.2
	N	337	63	92	95
Lateral Follicle Diameter	Median	104	153	165.9	131.2
	Range	331.8	287	199.2	204.7
	N	398	59	80	115
Midline Luminal Diameter	Median	50.2	156.4	61.5	71.7
	Range	238.7	322	168.3	188.9
	N	337	63	92	95
Lateral Luminal Diameter	Median	46.8	152.7	75.8	69.7
	Range	310.6	308.7	191.1	195.16

	N	398	59	80	115
Midline Number Cells	Median	4	4	5	4
	Range	16	12	17	8
	N	337	63	92	95
Lateral Number Cells	Median	3.5	3	4	4
	Range	15	8	8	8
	N	398	59	80	115
Midline Number Germinative Cells	Median	2	1	1	1
	Range	7	3	2	2
	N	337	63	92	95
Lateral Number Germinative Cells	Median	1	1	1	1
	Range	9	2	2	3
	N	398	59	80	115
Midline Number Intermediate Cells	Median	2	2	2	2
	Range	11	7	14	5
	N	337	63	92	95
Lateral Number Intermediate Cells	Median	1	2	2	2
	Range	10	7	7	5
	N	398	59	80	115
Midline Number Secretory Cells	Median	1	0	1	1
	Range	7	5	11	5
	N	337	63	92	95
Lateral Number Secretory Cells	Median	1	0	0	1
	Range	6	4	4	5
	N	398	59	80	115

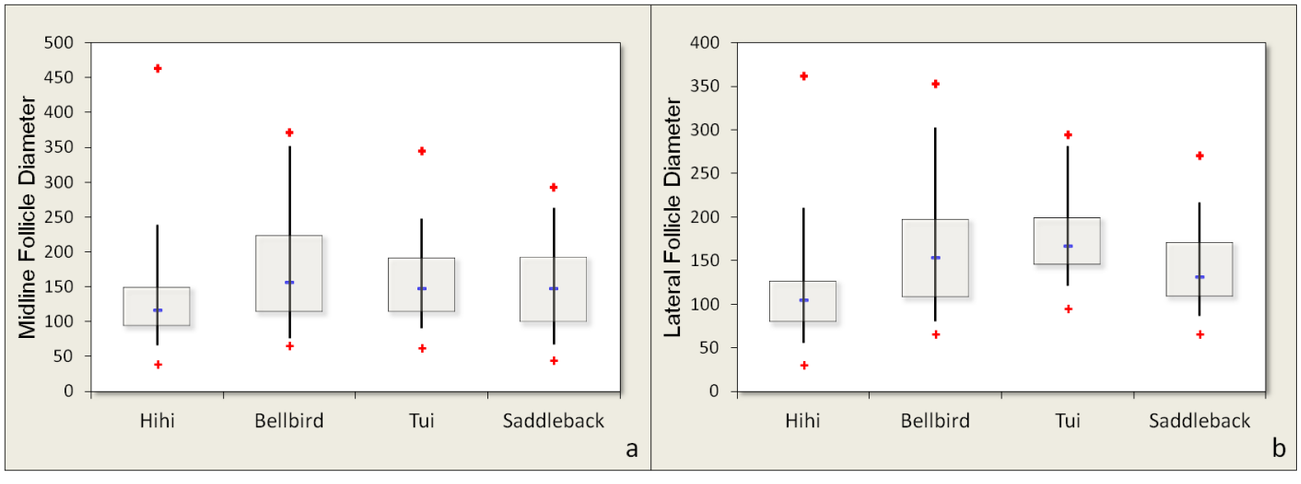

Figure 3.10: Showing UG follicle diameter of each species. a) Midline follicle diameter; b) lateral follicle diameter. Red dots = maximum and minimum values; dash in middle of box = median; top and bottom of box = upper and lower quartiles respectively.

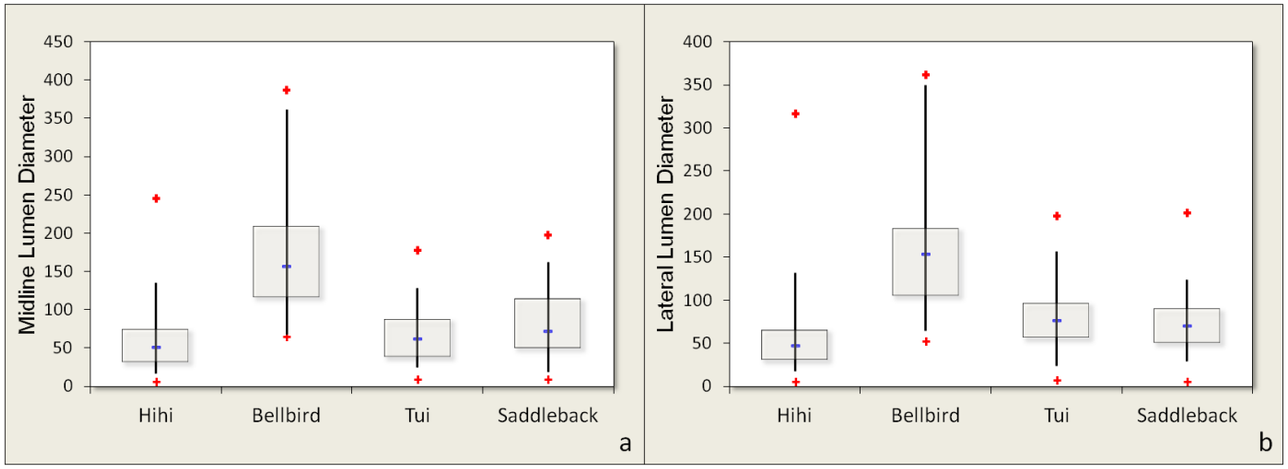

Figure 3.11: Showing UG lumen diameter of each species. a) Midline lumen diameter; b) lateral lumen diameter. Red dots = maximum and minimum values; dash in middle of box = median; top and bottom of box = upper and lower quartiles respectively.

The number of cells in the follicular epithelium were significantly different between species in both the midline (Kruskal-Wallis; *H*=17.356; df=3; p=0.001; Figure 3.12a; Table 3.3) and lateral sections (Kruskal-Wallis; *H*=16.239; df=3; p=0.001; Figure 3.12b; Table 3.3). Hihi possessed more germinative cells in their follicular epithelium compared to the other three species which possessed similar amounts (Figure 3.13; Table 3.3). Thus, a significant difference occurred between species in the number of germinative cells present in both the midline (Kruskal-Wallis; *H*=35.471; df=3; p=0.000; Figure 3.13a; Table 3.3) and lateral sections (Kruskal-Wallis; *H*=14.465; df=3; p=0.002; Figure 3.13b; Table 3.3).

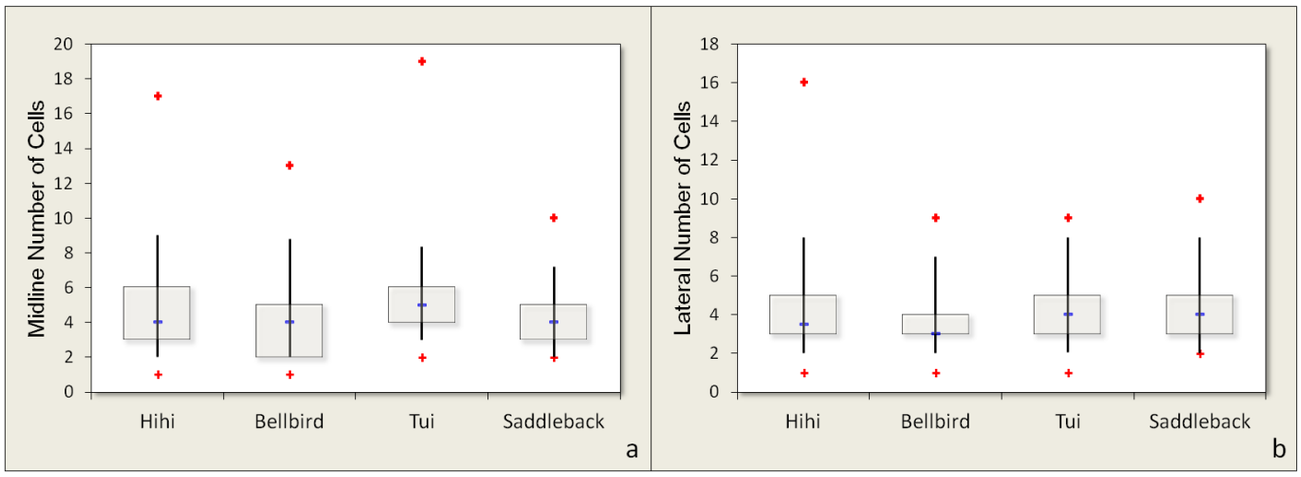

Figure 3.12: Showing the number of cells in the UG follicular epithelium of each species. a) Number of cells in the follicular epithelium of the midline of the UG; b) number of cells in the follicular epithelium of the lateral aspect of the UG. Red dots = maximum and minimum values; dash in middle of box = median; top and bottom of box = upper and lower quartiles respectively.

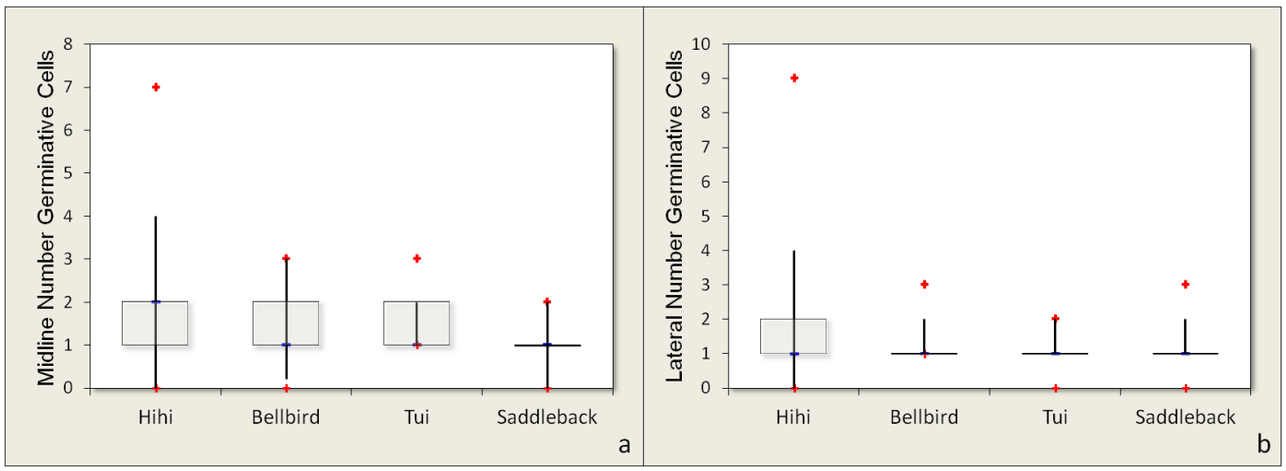

Figure 3.13: Showing the number of germinative cells in the UG follicular epithelium of each species. a) Number of germinative cells in the follicular epithelium of the midline of the UG; b) number of germinative cells in the follicular epithelium of the lateral aspect of the UG. Red dots = maximum and minimum values; dash in middle of box = median; top and bottom of box = upper and lower quartiles respectively.

Significant differences in the number of intermediate cells found in the follicular epithelium were found between species in the midline (Kruskal-Wallis; *H*=27.701; df=3; p=0.000; Figure 3.14a; Table 3.3) and lateral sections (Kruskal-Wallis; *H*=30.991; df=3; p=0.000; Figure 3.14b; Table 3.3) and also in the number of secretory cells in both

the midline (Kruskal-Wallis; *H*=14.582; df=3; p=0.002; Figure 3.15a; Table 3.3) and lateral sections (Kruskal-Wallis; *H*=37.958; df=3; p=0.000; Figure 3.15b; Table 3.3).

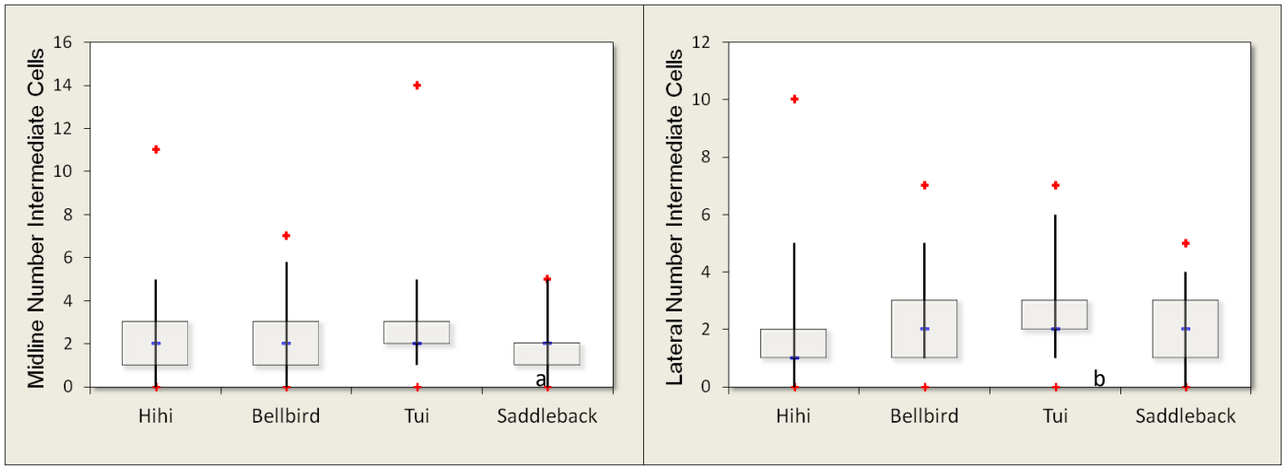

Figure 3.14: Showing the number of intermediate cells in the UG follicular epithelium of each species. a) Number of intermediate cells in the follicular epithelium of the midline of the UG; b) number of intermediate cells in the follicular epithelium of the lateral aspect of the UG. Red dots = maximum and minimum values; dash in middle of box = median; top and bottom of box = upper and lower quartiles respectively.

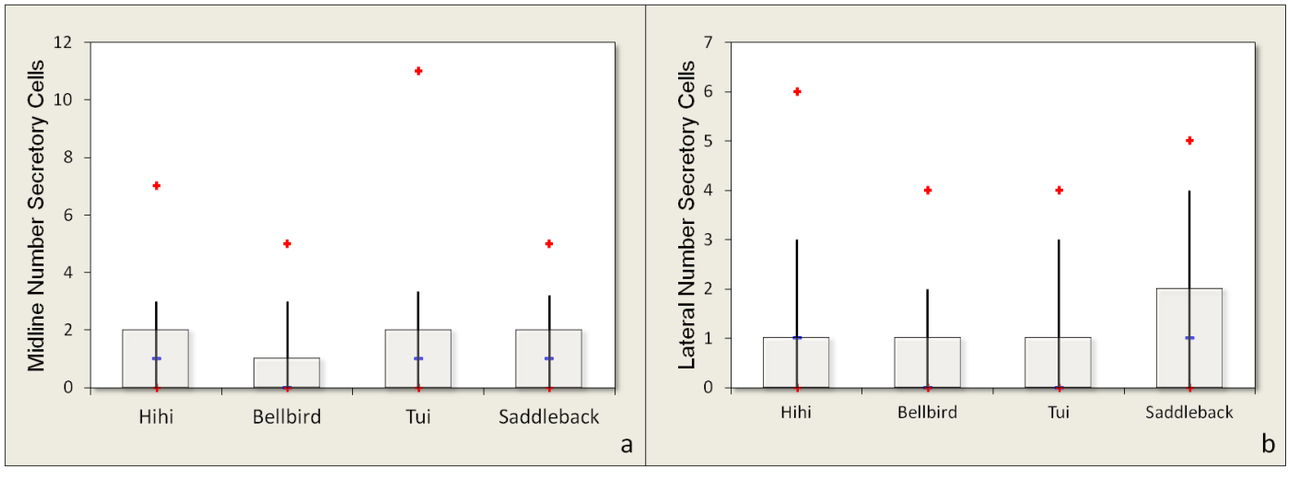

Figure 3.15: Showing the number of secretory cells in the UG follicular epithelium of each species. a) Number of secretory cells in the follicular epithelium of the midline of the UG; b) number of secretory cells in the follicular epithelium of the lateral aspect of the UG. Red dots = maximum and minimum values; dash in middle of box = median; top and bottom of box = upper and lower quartiles respectively.

3.4 Discussion

Each of the UGs in the four species investigated possessed features distinct to the order Passeriformes – they had two lobes each with a 'wart-like' papilla (Jacob & Ziswiler, 1982); there was a sharp distinction between the lobes and where the papilla arose from them, with the papilla most commonly making a right angle to the plane of the lobes; and there was a valve system that controlled the backflow of secretion from papillae to each lobe. These features match those found by Jacob and Ziswiler (1982) in the majority of passerines they studied (Table 3.4). Since their study there has been little work conducted on the UGs of other passerine species, despite their abundance throughout the world. Consequently there is limited histological information on this gland, especially with regards to the number of primary and secondary sinuses within each lobe.

When comparing the UG of these four New Zealand passerines to those studied by Jacob and Ziswiler (1982), I found that the New Zealand species all grouped together, as shown in table 3.4. They all possessed rather large glands when the ratio of lobe length to lobe width was compared (Table 3.4). The New Zealand bellbird possessed the largest gland of all four study species and this was surprising considering the small size of the bird (Heather & Robertson, 1994). However, the species above it on the table, the Hawfinch (*Coccothraustes coccothraustes*), is also a small terrestrial bird, indicating that a large UG can occur in these types of passerines. Taxonomically, it is interesting that the hihi, bellbird, tui, and saddleback sit so close to one another on the table, which would support the ancient radiation of these birds from a common ancestor in New Zealand when it first split from Gondwana (Ewen *et al.* 2006).

Table 3.4: Gross morphological characteristics and proportions of the UG in Passeriformes. Papilla direction: right angle = the papilla is almost at right angles to the plane of the lobes; rectilinear = the papilla barely makes an obtuse angle with the lobes. Ecology describes where the species most commonly inhabits. Species are listed according to the length:width index. Study species highlighted in grey. (Modified table from Jacob & Ziswiler, 1982).

Species	Index (Length/ Width)	Index (Lobe Length/Pap-illa Length)	Papilla Form	Papilla Direction	Ecology
Carduelis chloris	2.5	3.3	wartlike	right angle	Terrestrial
Vidua macroura	2.1	1.8	wartlike	right angle	Terrestrial
Coccothraustes coccothraustes	1.8	3.5	wartlike	right angle	Terrestrial
Anthornis melanura	1.8	4.1	wartlike	right angle	Terrestrial
Prosthemadera novaeseelandiae	1.7	4.6	wartlike	right angle	Terrestrial
Philesturnus carunculatus	1.7	4.5	wartlike	right angle	Terrestrial
Passer montanus	1.7	3.3	wartlike	right angle	Terrestrial
Notiomystis cincta	1.6	6.6	wartlike	right angle	Terrestrial
Fringilla coelebs	1.5	2.3	wartlike	right angle	Terrestrial
Aegithalos caudatus	1.5	3.3	wartlike	right angle	Terrestrial
Parus ater	1.4	4.3	wartlike	right angle	Terrestrial
Sitta europaea	1.3	5	wartlike	right angle	Terrestrial
Sturnus vulgaris	1.3	1.7	wartlike	right angle	Terrestrial
Euplectes orix	1.3	3	wartlike	right angle	Wetlands
Corvus corone	1.3	4.1	wartlike	right angle	Terrestrial
Nucifraga caryocatactes	1.3	4.9	conical	rectilinear	Terrestrial
Cardinalis cardinalis	1.3	2.2	wartlike	right angle	Terrestrial & Swamp
Pyrrhocorax graculus	1.2	4.2	conical	rectilinear	Terrestrial Alpine
Corvus monedula	1.2	4.2	wartlike	right angle	Terrestrial
Alauda arvensis	1.2	5.4	wartlike	right angle	Terrestrial
Hirundo rustica	1.2	6.3	wartlike	right angle	Terrestrial
Prunella modularis	1.1	5.7	wartlike	right angle	Terrestrial
Erythrura cyaneovirens	1.1	1.8	wartlike	right angle	Terrestrial Tropical
Corvus corax	1.1	4	wartlike	right angle	Terrestrial
Pica pica	1.1	5	wartlike	right angle	Terrestrial
Garrulus glandarius	1.1	4.5	conical	rectilinear	Terrestrial
Emberiza hortulana	1.1	2	wartlike	right angle	Terrestrial
Sporophila albogularis	1	2.5	wartlike	right angle	Terrestrial Tropical
Motacilla alba	1	5.6	wartlike	right angle	Terrestrial
Troglodytes troglodytes	1	3	wartlike	right angle	Terrestrial
Parus major	0.9	3.8	wartlike	right angle	Terrestrial
Hypargos niveoguttatus	0.9	2.5	wartlike	right angle	Terrestrial Sub-saharan
Erythrura prasina	0.9	3.1	wartlike	right angle	Terrestrial Tropical
Erithacus rubecula	0.9	2.2	wartlike	right angle	Terrestrail

Turdus philomelos	0.8	5	wartlike	right angle	Terrestrail
Erythrura trichroa	0.8	2.6	wartlike	right angle	Terrestrial Tropical
Emblema oculata	0.8	2.7	wartlike	right angle	Terrestrial
Poephila guttata	0.7	3	wartlike	right angle	Terrestrial

Although the four species studied appeared similar externally, the internal structure of the hihi, New Zealand bellbird, tui, and saddleback UG had substantial differences. Hihi, tui, and saddleback each possessed three primary sinuses while the bellbird had four clearly defined areas. In comparison to other avian species this is an unusual finding, as the most common UG has only two primary sinuses (Lucas & Stettenheim, 1972; Jacob & Ziswiler, 1982). It is interesting that the number of primary sinuses differed in the bellbird compared to the other three species and the papilla expressed peculiar attributes as well. Although they all possessed the valve system typical of the passerine UG, the bellbird's papilla contained much more connective tissue than that seen in the other three birds, which were more delicate in nature. This may have implications for the amount of UG secretion that could be stored within the bellbird papilla, as there would be less space. Consequently the bellbird may obtain less secretion from the gland during preening bouts and therefore their UG may be larger compared to the other three species to accommodate storage of more secretion.

Sadoon (2011) investigated the UG of the European starling and found that, like the hihi, bellbird, tui and saddleback, it was surrounded by a connective tissue capsule. This capsule was devoid of any muscle fibres and the gland itself sat on the base of the tail (Sadoon, 2011). The UGs of hihi, bellbird, and tui in this investigation were found to lie across the base of the tail in a similar fashion. Because of this location, Jacob and Ziswiler (1982) suggested that tail movement may have an indirect effect on the expulsion of secretion from the UG due to contraction of skeletal muscle associated with the tail. Follicles in the UG of hihi, bellbirds, tui and saddleback seemed to radiate out from the centre and end blindly toward the capsule. This arrangement was also found in starlings, moorhen, and pigeons (Bhattacharyya & Sahu, 1976). Interfollicular septae lay close to the basal cell

layers of follicles and did not cause the formation of discrete units of follicles as seen in other species, such as the rock ptarmigan (*Lagopus mutus*) and the Indian peafowl (*Pavo cristatus*) (Jacob & Ziswiler, 1982).

The cellular characteristics of the UG follicles in hihi and saddleback were similar to Sawad's (2006) findings in the osprey and Sadoon's (2011) findings in European starlings. The intermediate layer was thinner than that seen in other species; for example in the chicken (Gallus gallus domesticus) (Lucas & Stettenheim, 1972), and the secretory layer predominated. This may indicate a high level of lipogenesis (Sadoon, 2011) and therefore a larger production of secretion. Saddleback had the highest median number of secretory cells within the periphery of the gland, so secretion production may be highest in this area. Secretion production in the hihi may be more evenly distributed over the whole gland, as the hihi had similar numbers of secretory cells in both areas of the gland.

Bellbirds and tui were found to have more intermediate cells than secretory cells within their follicles. This suggests they may not be producing as much secretion as the hihi and saddleback. Alternatively, if the UG secretion volume changes seasonally, this difference could be a reflection of the time of year that the bird died. This feature may also be a characteristic of the family Meliphagidae or be the result of adaptations to particular ecological settings. Future studies could examine the histology of the UG of passerines at particular times of the year to investigate whether or not this has an influence on cell structure of the follicle. Future studies should also include more meliphagid species within the analysis to investigate whether they possess more intermediate cells than secretory cells in their follicular epithelium.

When considering the size of the UG in relation to the size of the bird, it was interesting to find that the tui and saddleback follicle diameters were not larger than the bellbirds. Hihi possessed the smallest follicle diameter and the smallest lumen diameter. Because the hihi is smaller in size than the tui and saddleback this result was logical. However, it would be expected that the bellbird also had small follicles but this was not the case. Larger follicles

may be a significant characteristic of this species suggesting there may be differences in the volume and composition of their secretion.

This investigation has unveiled important morphological characteristics of some New Zealand passerines. The UG appears to be similar at the species level. Examination of more passerine species would confirm this as at the moment there is a substantial deficit of information in regard to the UG's of Passeriformes. It would also be interesting to examine the UG of honeyeaters from Australia and the Pacific to compare their characteristics to those of the four New Zealand species currently studied. Because Jacob and Ziswiler (1982) did not include any honeyeaters in their study it is hard to reach firm conclusions regarding the differences between the UG characteristics of the bellbird and tui compared to those of the hihi and saddleback.

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Chapter 4: Effect of Sex and Season on the Cellular Topography of the Uropygial Gland of Hihi (*Notiomystis cincta*)

ABSTRACT

The uropygial gland is an integumentary gland of birds and is used during preening. There are four main hypotheses elucidating the function of the uropygial gland secretion: the feather maintenance hypothesis, the water-proofing hypothesis, the intraspecific communication/health hypothesis, and the defence against predators and parasites hypothesis. This chapter investigates facets of these hypotheses and focuses on the effects of season and sex of the bird on the cellular composition of the uropygial gland. We investigate hihi (*Notiomystis cincta*), a sexually dimorphic endemic New Zealand passerine. The aim was to find out if this sexual dimorphism extended to the uropygial gland. No significant effects of sex or season were found on several cellular characteristics of the hihi uropygial gland with exception of the lumen diameter, which was significantly larger in the winter than in the summer for both sexes. This bird may therefore produce more secretion in the winter months when compared to summer.

4.1 Introduction

Histology and several other techniques can be used to uncover information about the function of an organ; in this regard it is surprising to find that only 0.2% of all birds have had the histology of their uropygial gland (UG) investigated (Jacob & Ziswiler, 1982; Martin-Vivaldi *et al.* 2009; Harem *et al.* 2010). The UG produces oils and has several hypotheses proposed for its function, but to date there is no consensus as to what the function of the secretion is. The four main hypotheses regarding the use of the UG secretion by birds are: the feather maintenance hypothesis, the water-proofing hypothesis, the intraspecific communication/health hypothesis, and the defence against predators and parasites hypothesis (see chapter 1; Martín-Vivaldi *et al.* 2009; Salibian & Montalti, 2009).

Some predictions that arise from these hypotheses are based around aspects of seasonal change and sex differences. For example, depending on the gland's function, the composition and/or amount of secretion needed and produced by the gland may vary between seasons and/or between the sexes. In the hoopoe (*Upupa epops*) both seasonal and sex differences in the amount of UG secretion produced have been found. Female hoopoes have a drastic increase in the volume of secretion produced in the breeding season compared to non-breeding females and males; the secretion contains a mutualistic bacterium that, when secreted into the nest, out-competes pathogenic microorganisms, thereby protecting the young from disease and death (Martin-Vivaldi *et al.* 2009). In the mallard (*Anas platyrhynchos*) Kolattukudy and collegues in 1987 discovered differences in the composition of the UG secretion between breeding and non-breeding seasons. Female mallards had a UG secretion containing wax esters with short chain acids for the majority of the year, but during the breeding season the composition changed. Diesters of 3-hydroxy fatty acids were incorperated into the UG secretion and were belived to play a similar function to pheromones in other animals, as male mallards had no such change in their UG secretion composition. Male mallards respond to the change in composition by courting females with diesters of 3-hydroxy fatty acids in the secretion.

A recent study has shown that for several species of birds in New Zealand (e.g. South Island robin (*Petroica a. australis*), yellowhammer (*Emberiza citrinella*), and blackbird (*Turdus merula*)), the UG secretion varies between the breeding and non-breeding seasons (Fluen, 2008) although the reasons and consequences of this are unknown. This chapter aims to investigate the effects of season and sex on the cellular topography of the UG of hihi (*Notiomystis cincta*), a sexually dimorphic, endemic New Zealand passerine.

4.2 Materials and Methods

4.2.1 Study Species

Dead hihi were sourced from Massey University's Wildlife Health Centre from the archived collection under NZ DoC permit WE-3220-RES. Ten hihi were used in the study, five females and five males, which also represented five birds that died in summer and five birds that died in winter.

4.2.2 Histological examination

Each gland was sectioned sagitally into three slices. Initially, a section was made down the midline of the gland between the two papillae. One side of the gland was then used to produce three sections – a midline section, and two lateral sections (lateral 1 and 2) (see Figure 3.1a & b).

Each section was embedded in Leica Histo Embedder (Leica Paraplast® Tissue Embedding Medium (Melting Point 56ºC)) and cut to 4 micrometres in size using a Leica RM2235 Manual Rotary Microtome with a S35 Feather Microtome Blade (stainless steel). Sections containing bone were decalcified before being cut to avoid tearing the glandular region of tissue. Decalcification (the process of inorganic calcium removal) consisted of sections being soaked in osteomoll (Merck) overnight prior to processing. Each tissue section on their slide (Menzel-Glaser Superfrost PLUS Slides 76 x 26mm Ground edge 90º) was then routinely stained with Haematoxylin and Eosin (H&E) and was coverslipped wet via a xylene bath (Leica CV 5030 robotic coverslipper; Coverslips = Menzel-Glaser

22 x 50 No. 1; Mountant = Entellan ® rapid mounting medium for microscopy). Once dry, slides were examined under a light microscope with measurements of the glandular area being made with a Colourview digital camera and an AnalySIS 5 soft-imaging analysis system.

4.2.3 Histomorphometrics

For each section a longitudinal transect representing the maximum glandular diameter was made at 4x magnification (see Figure 3.2). The number of follicles along this transect were counted in order to obtain a measure of follicular density. The mean follicular diameter and mean luminal diameter of each follicle along the transect were measured at 40x magnification. The number and type of cells contributing to the follicle cell layer were recorded. The cell types in the hihi were classified into three zones labelled 'germinative', 'intermediate', and 'secretory'; refer to chapter three section 3.3.2 for cellular descriptions.

4.2.4 Statistical analysis

Paired t-tests were used to examine the effect of sex (male vs. female) and season (summer vs. winter) on mean follicular diameter, mean luminal diameter, number of cells in the follicular epithelium, number of germinative cells in a follicle cell layer, number of intermediate cells in a follicle cell layer, and number of secretory cells in a follicle cell layer.

All statistics are given to three decimal places. A Bonferroni corrected p value of 0.0042 was used. The reason for this is that when many tests on a set of data are performed, the null hypothesis is more likely to be rejected when it is true ("Type I" error) than when it is false. One strategy is to guard against making a mistake is to make the alpha level more stringent. This is achieved, for example, through calculating the Bonferroni corrected p value or 0.05/number of tests performed.

4.3 Results

4.3.1 Histomorphometrics

After Bonferroni correction there were no significant differences in any of the measured characteristics between male and female hihi or between summer and winter (Table 4.1). However, mean luminal diameter tended to be greater in birds that died in winter (Table 4.1; Figure 4.1).

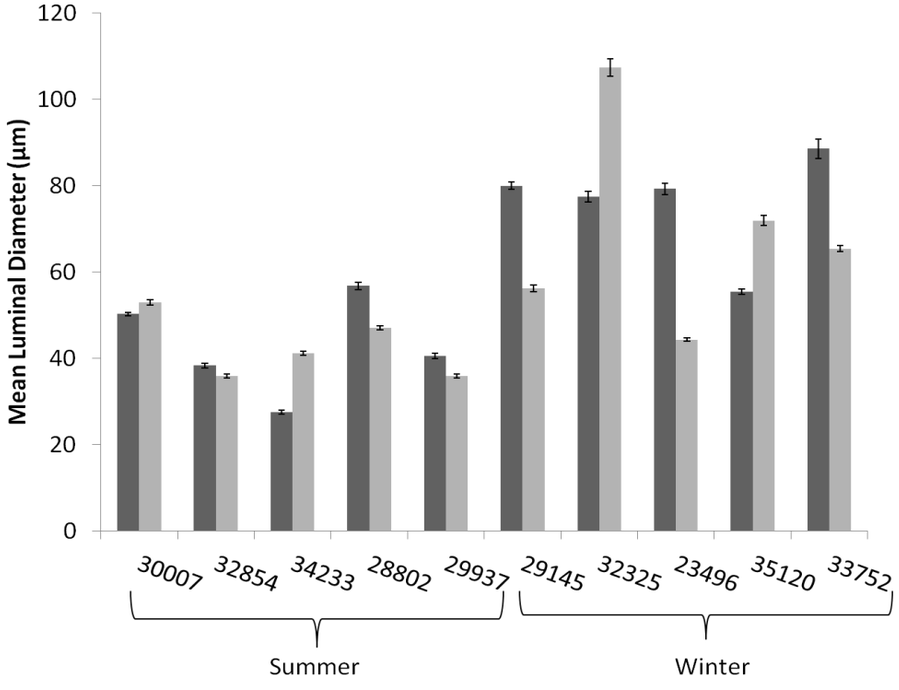

Figure 4.1: Mean luminal diameter of hihi uropygial glands (micrometers). Significant difference in season between midline only. Error bars represent standard error. Dark grey = midline section; light grey = lateral section.

Table 4.1: Paired t-test statistics for UG follicular characteristics of hihi. Comparisons of sex (male vs. female) and season (summer vs. winter) are shown; df = degrees of freedom, p = p-value. 12 tests so p Bonferroni corrected = 0.05/12 =0.0042

Sex	Paired t-test	Season	Paired t-test
Midline Follicle Diameter	t statistic = 0.303	**Midline Follicle Diameter**	t statistic = 2.440
	df = 4		df = 4
	p = 0.777		p = 0.071
Lateral Follicle Diameter	t statistic = 0.329	**Lateral Follicle Diameter**	t statistic = 0.794
	df = 4		df = 4
	p = 0.759		p = 0.472
Midline Luminal Diameter	t statistic = 1.077	**Midline Luminal Diameter**	t statistic = 3.529
	df = 4		df = 4
	p = 0.342		p = 0.024
Lateral Luminal Diameter	t statistic = 0.404	**Lateral Luminal Diameter**	t statistic = 2.119
	df = 4		df = 4
	p = 0.697		p = 0.102
Midline Number Cells	t statistic = 0.045	**Midline Number Cells**	t statistic = 0.536
	df = 4		df = 4
	p = 0.966		p = 0.620
Lateral Number Cells	t statistic = 0.180	**Lateral Number Cells**	t statistic = 0.154
	df = 4		df = 4
	p = 0.866		p = 0.885
Midline Number Germinative Cells	t statistic = 0.530	**Midline Number Germinative Cells**	t statistic = 0.677
	df = 4		df = 4
	p = 0.624		p = 0.536
Lateral Number Germinative Cells	t statistic = 0.164	**Lateral Number Germinative Cells**	t statistic = 0.967
	df = 4		df = 4
	p = 0.878		p = 0.388
Midline Number Intermediate Cells	t statistic = 0.021	**Midline Number Intermediate Cells**	t statistic = 0.230
	df = 4		df = 4
	p = 0.984		p = 0.829
Lateral Number Intermediate Cells	t statistic = 0.708	**Lateral Number Intermediate Cells**	t statistic = 0.353
	df = 4		df = 4
	p = 0.518		p = 0.742
Midline Number Secretory Cells	t statistic = 1.191	**Midline Number Secretory Cells**	t statistic = 0.243
	df = 4		df = 4
	p = 0.300		p = 0.820
Lateral Number Secretory Cells	t statistic = 0.234	**Lateral Number Secretory Cells**	t statistic = 0.614
	df = 4		df = 4
	p = 0.827		p = 0.572

4.4 Discussion

No significant effects of sex or season were found on several cellular characteristics of the hihi UG. There was a tendency for the lumen diameter to be larger in the winter than in the summer, although after Bonferroni correction for multiple tests, the difference was not significant. However, a larger lumen could indicate that the UG secretion synthesis (and in turn preening) is increased in the winter months due to environmental pressures such as severe/colder weather, which may promote the need for preening. Lumen diameter, therefore, would be larger in winter to accommodate the increase in secretion and demand for preening. A study by Spinu, Benveneste, & Degen (2003) showed that preening activity increased in winter compared to summer in White Rock broiler breeding hens (*Gallus gallus domesticus*).

Some studies claim there are differences in the composition of the secretion between seasons (Kolattukudy *et al.* 1987). Cells can alter the secretory products they produce through changes in biochemical processes controlled by the nucleus (Marieb & Hoehn, 2007). Perhaps future studies need to delve deeper into the histochemistry of the cells to uncover differences in what they produce between seasons, as it may be that *cell content* and not the *number of cells* in the follicle cell layer determines secretion differences.

In a few species (such as the European hoopoe (*Upupa epops*)) the external characteristics of the UG have been shown to differ between sexes but it has never been shown histologically (Martin-Vivaldi *et al.* 2009). Breeding and nestling female European hoopoes possess a dark and pungent UG secretion, as well as UGs eight-fold larger than that seen in males and non-breeding females of the same species (Martin-Vivaldi *et al.* 2009). This is the only bird known to have external differences between sexes in the UG structure. Unfortunately there is no information on the histology of the gland in this species, for this would provide an insight into any differences between cellular structure in the breeding female and male glands. Future investigations could compare UG cellular characteristics between the sexes as this may lead to discoveries in compositional differences.

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Chapter 5: The Examination of Ectoparasite (*Ixodes anatis*) Behaviour Toward the Uropygial Gland Secretion of Brown Kiwi (*Apteryx mantelli*).

ABSTRACT

Ectoparasites frequently survive in association with animals. Within birds, associations with some ectoparasites cause decreases in fitness of the hosts, resulting in the evolution of defensive behaviours. In addition, physiological mechanisms, such as the secretion of the uropygial gland, have been posed as defence mechanisms against ectoparasites. Brown kiwi (*Apteryx mantelli*) are home to a variety of ectoparasites, including the kiwi tick (*Ixodes anatis*). These birds roost and nest in underground burrows and on Ponui Island the population of brown kiwi is very high. Therefore, the kiwi tick population is maintained at high levels due to the high numbers of the birds and their habits. As a result an ectoparasite defence mechanism would be advantageous for this population.

Ticks were exposed to each oil (2cm x 2cm square in a Petri dish) in turn and to a control (Petri dish with no oil). Their behaviour toward the substance was recorded – whether they remained within the square of oil or whether they left. Results indicated a significant difference in the behaviour of the ticks between oil treatments and the control. Engorged ticks are most often attached to the host's body and locomotion is restricted, whereas, non-engorged ticks (unattached to the host's body) are unresitricted in their movements. All non-engorged ticks left the square of uropygial gland oil. All males, 70% of females, and 50% of the nymphs left the square of uropygial gland secretion. These results suggest engorged female ticks dislike the UG secretion more than nymphs and, because male ticks are always on the search for receptive females, they may have simply left the square of oil

because they are more active than the other stages, in the sense that finding receptive females requires their active movement around the host.

Overall we can hypothesize that the tick species *I. anatis* may dislike oily substances and birds could use the uropygial gland secretion as a means of controlling ticks. The UG secretion may promote detachment of ticks from the birds' plumage, and if this occurs at inadequate times of the ticks' life cycle, it may cause death of the ticks and thus reduce or change re-infection patterns.

5.1 Introduction

Bird-ectoparasite associations are frequent in the animal kingdom. Many of these associations cause a loss in fitness of the host (Moller, 1991; Heath, 1994, Proctor & Owens, 2000; Moller, Erritzoe, & Rozsa, 2010). Thus many strategies have evolved to reduce ectoparasite loads (Moller, Erritzoe, & Rozsa, 2010). Ectoparasites are arthropods which parasitize the outside of a host, for example, fleas (Siphonaptera), lice (Phthiraptera), flies (Diptera), true bugs (Hemiptera), and ticks and mites (Acari) (Moyer & Clayton, 2004). The level of association between hosts and ectoparasites differs among species, for example, some ectoparasites complete their entire lifecycle on one host and some require more than one (Moyer & Clayton, 2004). Horizontal transmission of ectoparasites is common among a host species, especially in those individuals in close proximity to one another (Moyer & Clayton, 2004).

Resources exploited by ectoparasites vary greatly (Moyer & Clayton, 2004). The cattle tick (*Haemaphysalis longicornis*) feeds on the blood of the host; therefore penetration of the host's skin occurs (Anderson & Valenzuela, 2008). Feather lice (Phthiraptera), on the other hand, feed entirely on feathers, thus extreme feather loss can occur in highly infected individuals (Moyer & Clayton, 2004). Whatever the mode of exploitation it is well known that ectoparasites can have detrimental effects on host fitness. Reproduction, survival, mate choice, and fecundity are all aspects of life history that can be affected by ectoparasites (Moller, 1991; Heath, 1994, Proctor & Owens, 2000; Moller, Erritzoe, & Rozsa, 2010). Hamilton and Zuk (1982) argued that sexual selection could be biased towards the healthier individuals within a species i.e. the individual with the greatest resistance to a particular parasite. Mate choice in this instance is based on secondary sexual traits which indicate ectoparasite load, for example, courtship displays in which parasitized individuals are not able to perform (Moyer & Clayton, 2004) or more direct effects such as those caused by *Ornithonyssus bursa,* which shorten tail length in swallows (*Hirundo rustica*) (Moller, 1992).

Ectoparasites also pose direct harm to the bird, as they are known vectors for a variety of diseases (Reneerkens, 2007). Keeping ectoparasites at low numbers is therefore important to host survival and reproduction, and birds have evolved many defence mechanisms against them (Clayton *et al.* 2010). Each hypothesised defence system is expanded on in the following section.

5.1.1 Avian defence mechanisms against ectoparasites

Preening is the most obvious strategy used in ectoparasite defence. Preening occurs when the bird pulls feathers between the bill mandibles or nibbles feathers with the bill tip (Figure 5.1; Clayton *et al*. 2010). According to Goldstein (1988), energy costs associated with preening behaviours approximate to twice basic metabolic rate. Birds spend a large portion of their daily time preening, therefore, this behaviour is costly not only in terms of time but also in the amount of energy exerted by an individual (Losito, Mirarchi, & Baldassarre, 1990).

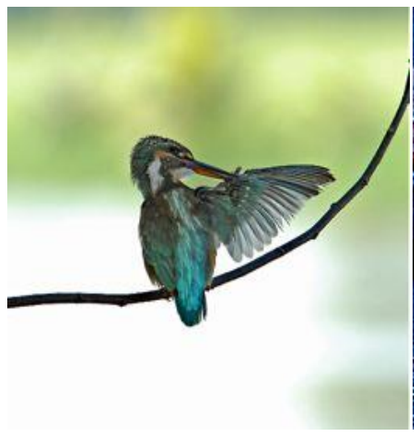

Figure 5.1: Photograph of a Common Kingfisher (Alcedo atthis) preening. (http://www.besgroup.org).

Figure 5.2: Picture illustrating the comb-like structure of the pectinate claw. (http://people.eku.edu/ritchisong/avian-_integument.htm).

Birds with bill deformations provided initial indications of the importance of preening in combating ectoparasites (Moyer & Clayton, 2004; Clayton *et al*. 2010). Brown (1972) altered domestic chicken (*Gallus gallus domesticus*)

bills by clipping the upper mandible. Clipped-billed birds showed elevated ectoparasite numbers compared to those with normal beaks. Later experiments impaired preening behaviours using *poultry bits* (small, C-shaped pieces of metal/plastic crimped in the nostrils to hold in place) (Booth, Clayton, & Block, 1993) which were inserted between the mandibles of the bill (Clayton *et al.* 1999). Results reinforced the importance of preening in maintaining ectoparasite loads at controllable levels (Clayton *et al.* 1999).

Scratching is another grooming behaviour used for controlling ectoparasite numbers (Moyer & Clayton, 2004; Clayton *et al.* 2010). This behaviour utilises the feet and occurs in places of the body where preening is not possible, for example the head. Scratching causes damage to ectoparasites, and from studies on fleas of domestic chickens it has also been shown to kill them (Suter, 1964, cited in Marshall, 1981, p. 107). When a bird lacks other methods of ectoparasite control they compensate by increasing the amount of time spent scratching. For example, a study by Brooke (1985) showed that unpaired penguins spent more time scratching compared to paired penguins that engaged in allopreening. A recent study by Clayton *et al.* (2010) investigated the possible role of the pectinate claw in its ability to increase efficiency of scratching to control ectoparasites. The pectinate claw is present on the middle toenail of some birds and has a comb-like appearance (Figure 5.2). Results from their investigation, however, imply that the claw serves no purpose in ectoparasite control.

Birds are also known to adopt a variety of behavioural defence mechanisms against ectoparasite infestations. Many species 'dust' themselves, a behaviour where the individual spreads dust, sand or other fine particles through its plumage (Simmons, 1985; Hendricks & Hendricks, 1995). Primarily, this behaviour is thought to maintain an appropriate amount of lipid on the plumage, since too much oil causes matting of the feathers (Healy & Thomas, 1973). Hoyle (1938) investigated dusting in the domestic chicken and noted that ectoparasites were dislodged from the body during the performance of the behaviour. Dusting is also injurious to ectoparasites as it causes desiccation through abrasion of the cuticle (Hendricks & Hendricks, 1995), poor respiration due to spiracle

blockage (Moyer & Clayton, 2004), and inadequate nutrition as some ectoparasites rely on plumage oil as food (Borchelt & Duncan, 1974).

Bathing in water has been speculated as a method of ectoparasite control. In disagreement, Clayton *et al.* (2010) suggested that bathing could have a positive effect on ectoparasite numbers. Ectoparasites thrive in humid environments (Moyer, Drown, & Clayton, 2002), thus bathing would cause the plumage habitat to increase in humidity, thereby offering ectoparasites a selective advantage. However, perhaps bathing hinders respiration in ectoparasites? It is possible (as with dusting behaviour) that the spiracles of the ectoparasite could become blocked, causing respiratory difficulty and eventually death. Birds could also seek out water with dissolved substances harmful to ectoparasites, as this would aid in their demise (Clayton *et al.* 2010).

Exposure to solar radiation while adopting stereotypical postures is known as 'sunning'. There are over 50 families of birds that sun themselves on a regular basis (Simmons, 1986). Over-heating and death of ectoparasites has been suggested as a possible reason for sunning behaviours (Moyer & Wagenbach, 1995). A study conducted by Moyer and Wagenbach (1995) gave evidence supporting over-heating as a means of ectoparasite death. They investigated the chewing louse, *Quadraceps hopkinsi,* which thrives on feathers of the Black Noddy (*Anous minutes*). Black Noddies sun gregariously, causing the temperature of the feathers to become exceedingly high (>29°C). Results revealed that the chewing lice could not endure the high temperatures, therefore sunning in this species resulted in the death of ectoparasites. Further, a more indirect means of ectoparasite control via sunning is through preening of ectoparasites as they try to escape the high temperatures of the plumage (Simmons, 1986). Although these are both plausible explanations, more work is required on other species of ectoparasite, as some maybe more resistant to temperature fluctuations than others.

'Anting' – the act of crushing and subsequently spreading ants through the plumage or allowing ants to crawl through the plumage – is another behavioural anti-parasitic mechanism (Moyer & Clayton, 2004). Over 200 avian species, mostly from the Passeriformes order, have been observed to ant (Clayton *et al.* 2010). Dubinin (1951) found that feather mites (*Pterodectes* spp) on the wing feathers of meadow pipits (*Anthus pratensis*) displayed extremely more locomotion across feathers of birds that had anted compared to mites on feathers of birds who had not. He also noted that mites in the 'moist' areas (i.e. where the ants had been smeared) were dead. However, compared to Dubinins (1951) account, the remaining literature disagrees, as little evidence supports his observations. Revis and Waller (2004) concluded that ant chemicals had no effect on the growth of specific feather bacteria and fungi; and Clayton *et al.* (2010) also found anting to have little effect on mites and lice of European starlings (*Sturnus vulgaris*). Thus, anting as an ectoparasite defence is controversial and more investigation is required as to its role.

Because ectoparasites inhabit the outside of the host, they are most commonly in contact with the plumage (Clayton *et al.* 2010). Therefore, just as behavioural defences against ectoparasites have evolved it is not unlikely that selective pressures have caused features of the bird's body to evolve ectoparasite deterrence properties as well. The feather moult has been suggested as a physiological control mechanism for ectoparasite infestations. This event rids immobile ectoparasites such as bacteria or fungi from the plumage; however this is speculative for mobile species such as ticks (Clayton *et al*. 2010). Hamstra and Badyaev (2009) suggest that ectoparasite numbers rise during moult. Results from their investigation showed an increase of the feather mite *Strelkoviacarus* spp and *Dermoglyphus* spp during feather moult of house finches (*Carpodacus mexicanus*). They credited this to the increase in energy costs associated with moulting, as this event reduced available energy for other activities such as preening. Conversely, many studies oppose this view and have evidence to support a drop in mobile ectoparasite numbers over the moulting period (see McGroarty & Dobson, 1974; Chandra *et al.* 1990).

The toughness of a feather is another barrier suggested to control ectoparasites. Melanin pigmented feathers (e.g. black, brown or grey) are more resistant to abrasive wear than non-melanin pigmented feathers (e.g. white) (Bonser, 1995; Clayton *et al.* 2010). Moller (1991) demonstrated that holes chewed on the tail feathers of swallows by the mite *Ornitthonyssus bursa* were more abundant in white regions compared to black regions of the tail feathers. Kose, Mand, and Moller (1999) later went on to support this by reporting that lice *Hirundoecus malleus* from barn swallows (*Hirundo rustica*) spend more time on white tail feathers than black in vitro. These studies not only support Hamilton and Zuks (1982) parasite-mediated mate choice hypothesis, but demonstrate that toughness associated with melanin gives feathers that acquire this pigment a resistance against feather-feeding ectoparasites.

Some birds harbour toxins in their feathers and skin – this too is considered an anti-parasitic strategy. Poison dart frogs (*Phyllobates* spp) contain noxious batrachotoxins in their skin which aid in deterring predators. In 1992 Dumbacher *et al.* discovered the presence of these toxins in the skin and feathers of several avian species of the genus *Pitohui*. Like the frog, this toxin is thought to play a role in deterring predators; however, it has recently also been suggested as a way of repelling ectoparasites (Moyer & Clayton, 2004). Dumbacher (1999) demonstrated experimentally that lice suffered mortality sooner when in contact with toxic feathers from *Pitohui dichrous* than when in contact with feathers from a non-toxic avian species (*Colluricincla megarhyncha*). This would be beneficial for the bird as it would reduce both the amount of time the parasite spends feeding on the host and the ectoparasite survival to sexual maturity.

Strong odours have been suggested as a chemical defence against ectoparasites (Clayton *et al.* 2010). Crested auklets (*Aethia cristatella*) produce a citrus smelling odour and its composition suggests it may possess defensive functions (Douglas, Malenke, & Clayton, 2005). Hexanal and octanal are known repellents of arthropods; they are both present in the secretion of the wick feathers of these birds and therefore they are key components of the

bird's odour. Douglas *et al.* (2005) tested this hypothesis by exposing rock pigeon lice (*Columbicola columbae* and *Campanulotes compar*) to fresh crested auklet feathers. There was no impact on lice survivorship and, furthermore, crested auklets were found to harbour more lice than the closely related least auklets – which do not produce an odour. However, when the authors exposed the odour to a tick species found on crested auklets in nature – *Ixodes uriae* – the ticks became moribund in less than fifteen minutes (Douglas *et al.* 2004). Controls in this experiment were still alive after two days, suggesting that the odour was a powerful repellent of some ectoparasites specific to crested auklets in nature (Douglas *et al*. 2004).

The uropygial gland (UG) – or preen gland – secretion is also hypothesised to combat ectoparasites. The UG is an integumentary gland present in birds (Moyer, Rock, & Clayton, 2003). The secretion produced is exceptionally oily and its composition has been found to be species specific (Martín-Vivaldi *et al.* 2009). The oil is spread through the plumage during preening and many hypotheses have been proposed to describe its function, including defence against ectoparasites (Elder, 1954; Jacob & Ziswiler, 1982). In support of the anti-parasitic hypothesis Moyer *et al.* (2003) showed how uropygial gland oil from rock doves (*Columba livia*) increased the mortality of lice (*Columbicola columbae* and *Campanulotes bidentatus compar*) *in vitro*. They exposed lice to feathers with and without uropygial gland oil, discovering that lice exposed to feathers with uropygial oil had increased mortality rates. Contrary to this result, the same authors found no effect of uropygial gland oil on lice numbers *in vivo,* suggesting that concentrations used in the *in vitro* experiments might not have accurately represented concentrations of secretion under natural conditions. Uropygial gland oil may not only have anti-parasitic properties, but its viscosity may affect ectoparasite survival. Because of its oily nature, ectoparasite mobility may be reduced on the plumage and skin. Similar to dusting and bathing, oiling may also block spiracles causing asphyxiation (Clayton *et al.* 2010).

Brown kiwi are highly scented birds that use underground burrows as roosts and nests. These burrows are shared by members of a pair and in areas of high density are used by several pairs sequentially (Ziesemann 2011). Brown

kiwi harbour a number of parasites, often in high numbers. Brown kiwi excreta and UG secretion has been found to contain octanal and decanal precursors and derivatives (Jacob 1982; Castro et al. 2010; Castro et al. in prep.). Ticks cause damage to their hosts in three direct ways. Mechanical damage occurs when the tick bites into the skin of the host (Heath, 1985; Heath, 1994). After penetration of the host's skin the tick injects saliva into the wound which actively dissolves tissue and prevents blood from coagulating. At this point, pharmacologically active substances enter along with saliva and may elicit an immune response in the host (Heath, 1985). The last direct effect of the tick on its host is the removal of blood (Neilson, 1980). This causes the host to become anaemic and, if untreated, energy reserves are depleted to exhaustion, causing death. An indirect effect of tick infestation is the transmittance of diseases through the injection of allergens, parasites, or removal of blood (Heath, 2010).

This investigation, therefore, focuses on the response of the kiwi tick – *Ixodes anatis* – to the UG secretion of brown kiwi in order to investigate whether the secretion acts as as an ectoparasite repellent.

5.2 Materials and methods

5.2.1 Study site

Tick samples for this study were collected from wild brown kiwi on the South Ponui Farm, Ponui Island in the Hauraki Gulf of New Zealand (Latitude 36°55'S, Longitude 175°11'E) in the months of May and June 2012 (Figure 4.1). This island is privately owned and is 1770 hectares in size. Three farms form the island (South, Central, and North) where pasture is the predominant landscape (Miles & Castro, 2000). Native forest covers the remainder of the island and primarily consists of regenerating Kauri (*Agathis australis*) forest and Kanuka (*Kunzea ericoides*), mixed with broadleaf forest and wetlands (Miles & Castro, 2000).

5.2.2 Study species

5.2.2.1 Brown kiwi (*Apteryx mantelli*)

Brown kiwi are flightless, endemic ratites of New Zealand and are considered to be 'seriously declining' by the Department of Conservation (Holzapfel *et al.* 2008). They are nocturnal and roost/nest in burrows, hollow logs, or dense scrub during the day (Heather & Robertson, 2005). Females are larger than males, and produce very large eggs (15-20% of body weight) that are generally incubated by the male (Heather & Robertson, 2005). Incubation is extended in this species and ranges from 74-84 days (Ziesemann, Brunton, & Castro, 2011).

Brown kiwi possess an entourage of ectoparasites (Table 5.1). In addition to kiwi-specific ectoparasites (for example *Ixodes anatis*), brown kiwi are known to carry adventitious ectoparasites (for example cattle ticks – *Haemaphysalis longicornis)* (Heath, 2010). Ponui Island, where the parasites for this study were obtained, has a dense kiwi population (Cunningham, Castro, & Alley, 2007) most likely comparable to those before the arrival of humans (Ziesemann, Brunton, & Castro, 2011) and the introduced mammals that have decimated kiwi populations elsewhere. This high density, together with the habit of kiwi to share nests and roosts with several other kiwi (Ziesemann, 2011), permits parasites to build numbers and move easily between hosts.

Table 5.1: Ectoparasites collected from brown kiwi (Apteryx mantelli) in New Zealand (Heath, 2010). indicates endemic to kiwi.*

Type of parasite	Species name	
Feather Mites	*Kiwialges palametrichus**	*Kiwialges phalagotrichus**
Chewing Lice	*Apterygon mirum**	*Rallicola (Aptericola) roderickі**
Fleas	*Pygiopsylla phiola*	*Nosopsyllus fasciatus* *Pulex irritans*
Ticks	*Ixodes anatis**	*Haemaphysalis longicornis*
Trombiculid Mites	*Guntheria (Derrickiella) apteryxi**	

Figure 5.3: Photograph of 'Mauro' – Ponui Island brown kiwi male. Photograph by Jay Bent.

5.2.2.2 *Ixodes anatis*

Ixodes anatis is an endemic ectoparasite of kiwi (Cane, 2009; Heath, 2010). They are distributed throughout New Zealand (Figure 5.4) although more kiwi populations need to be assessed to determine its exact occurrence (Heath, 2010). They are known to occur in large numbers on individual birds (Heath, 2010) and are most commonly found around the head region (Morgan, 2008). *I. anatis* is an endophilic nidicolous species – in other words they live in the burrow/nest of their hosts (Heath, 2010).

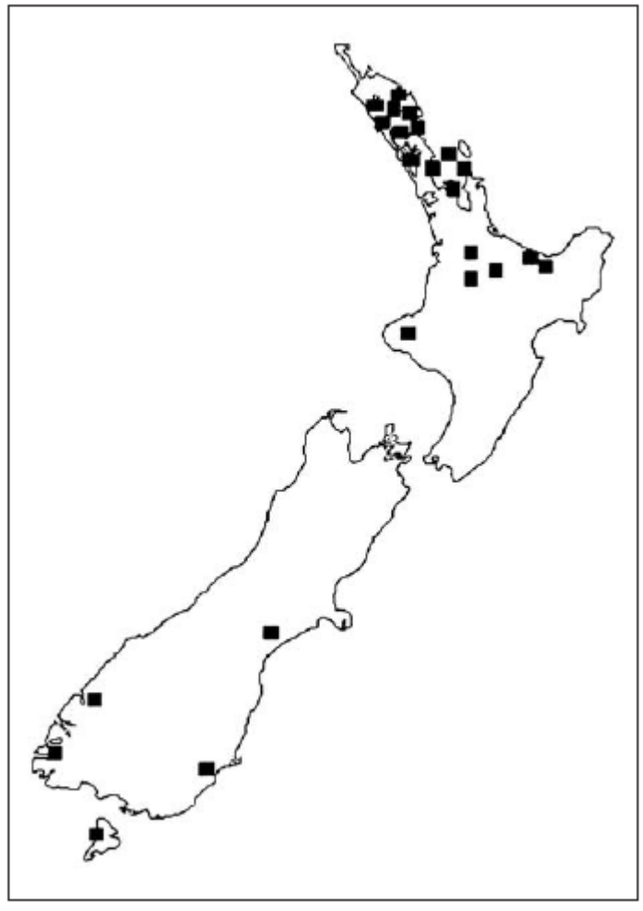

Figure 5.4: Distribution of Ixodes anatis within New Zealand (Heath, 2010).

Although evidence is lacking, Heath (2010) suggested that *I. anatis* is a three-host tick like most other ixodid species (see Figure 5.5). In ixodid lifecycles the tick spends times off the host at each life stage. Each host is commonly a different species (Wall & Shearer, 2001). However, due to *I. anatis's* endophilic nature, together with the fact that brown kiwi on Ponui often re-use burrows, it is likely that the same bird acts as the host for each life stage of the tick (Heath, 2010; Ziesemann, 2011). Therefore, re-use of burrows/nests in the Ponui population maintains the *I. anatis* population, as starvation is less likely between instars.

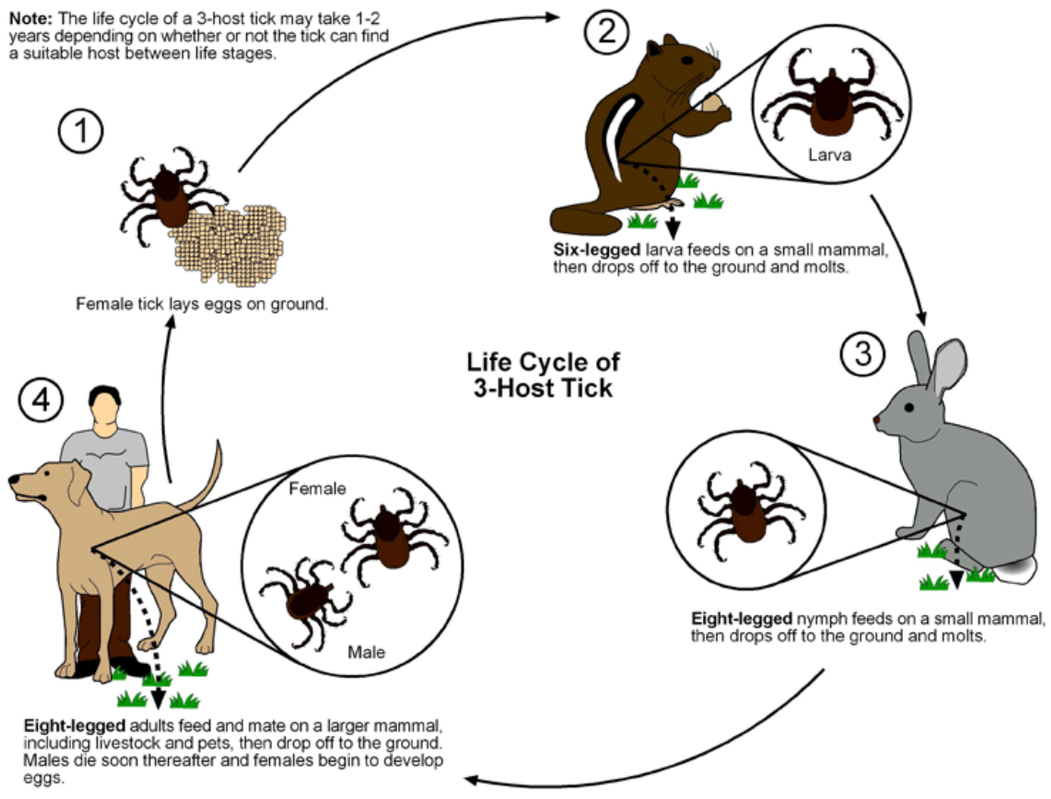

Figure 5.5: Ixodid tick three-host life-cycle (http://extension.entm.purdue.edu/publichealth/insects/tick.html)

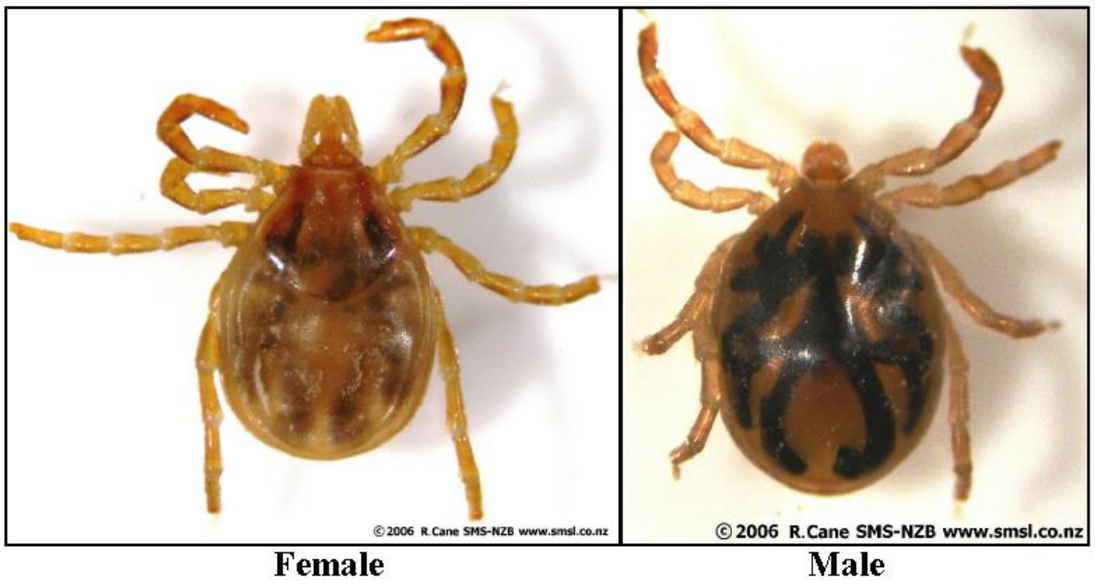

Figure 5.6: Photograph illustrating differences between unengorged female tick and male tick – Ixodes anatis (Cane, 2009).

5.2.3 Experimental procedure

Ticks were removed from birds by hand (unattached ticks) or carefully using tweezers (attached ticks) and were placed in small bottles kept warm in a thermos until the experiment started. Both ticks and UG secretion were collected in May and June 2012. UG secretion was collected from five adult brown kiwi. Kiwi were caught during the day after finding them via radio telemetry. After separating the feathers, the UG was stimulated to release secretion by a gentle massage. Approximately 20-50µL of UG secretion was collected in a capillary tube and transferred to a storage tube (see Figure 4.2). Secretion was collected under New Zealand Department of Conservation permit AK29244FAU and Animal Ethics Committee approval MUAEC 11/60.

Each tick was exposed to three different treatments: 1) control, 2) UG secretion, and 3) Homebrand® canola oil (Figure 5.7b). Treatments 2 and 3 had a 2cm by 2cm smear of oil (approximately 5 µL) applied to the bottom of a Petri dish using a capillary tube forming a square (Figure 5.7a). Ticks were placed in the middle of the oil square (for the control in the middle of the dish) and their movement within the dish was recorded for five minutes using a Sony Digital Video Camera Recorder (DCR-SR45) (Figure 5.7a & b).

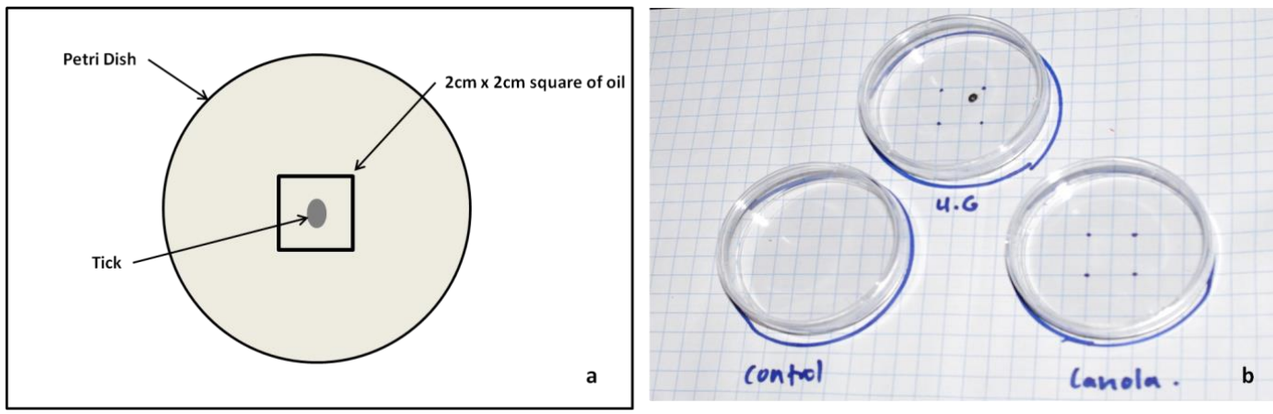

Figure 5.7: a) Illustration of experimental design showing Petri dish with square of oil and tick placed in the middle; b) Photograph of experimental setup showing three separate treatments: 1) control, 2) UG secretion, and 3) Homebrand® canola oil. Ticks were exposed to each treatment for 5 minutes. Illustration and photograph by author.

5.2.4 Statistical analysis

Fisher's exact tests and chi-square tests of association were employed to analyse the effect of sex, body condition, and life stage of the tick to the three treatments. These two tests were used because the data were categorical: male vs. female (sex – excludes nymphs); non-engorged vs. engorged (body condition); adult vs. nymph (life stage). Some of the chi-square tests had expected counts less than five, so the Fisher exact test replaced the chi-square tests in these instances as it is a more appropriate test for small sample sizes.

A probability of ≤0.05 was taken as significant.

5.3 Results

Of the ticks tested there were 28 nymphs, 23 female, and 16 males, giving a total of 67 specimens. There was a significant difference in the behaviour of the ticks between the oil treatments and the control (Figure 5.8). In the control, all ticks moved outside the central area, which corresponded to the position of the oil in the other treatments. The sex of the tick had a significant influence on its response to the UG secretion (Fisher's Exact Test, p=0.029). All male ticks left the square of UG secretion (Table 5.2). Fifty percent of nymphs remained within the square while the other fifty percent left. The association between sex and canola oil, however, was not significant at the 0.05 level (Fisher's Exact Test, p=0.066) (Table 5.3).

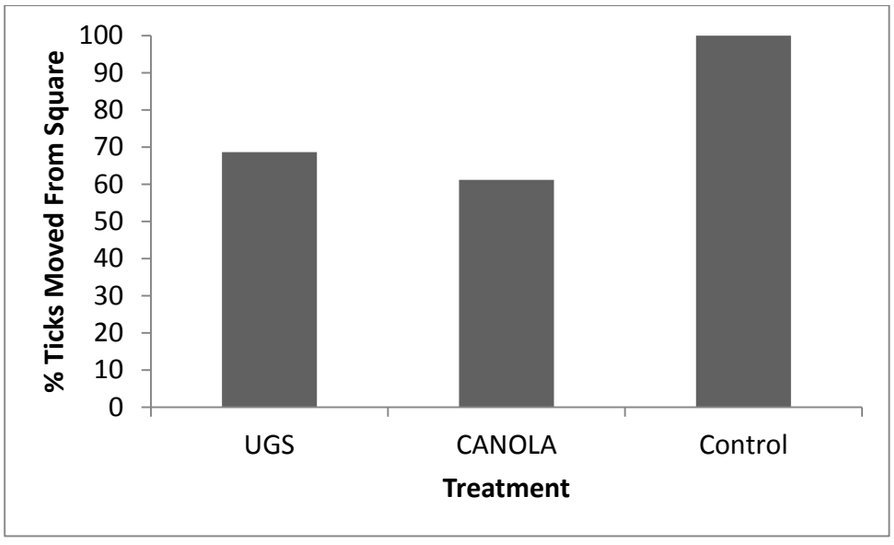

Figure 5.8: Figure showing the percentage of ticks which left the central square under each treatment (UGS = uropygial gland secretion, canola oil, and control).

Table 5.2: Cross tabulation of sex, body condition, life-stage and uropygial gland secretion

			Uropygial Gland Secretion	
			Leave	Stay
Sex	Female	Observed Count	16	7
		Expected Count	19	4
	Male	Observed Count	16	0
		Expected Count	13	3
Body Condition	Engorged	Observed Count	26	21
		Expected Count	32	15
	Non-engorged	Observed Count	20	0
		Expected Count	14	6
Life-stage	Adult	Observed Count	32	7
		Expected Count	27	12
	Nymph	Observed Count	14	14
		Expected Count	19	9

Table 5.3: Cross tabulation of sex, body condition, life-stage and canola oil

			Canola Oil	
			Leave	Stay
Sex	Female	Observed Count	18	5
		Expected Count	20	3
	Male	Observed Count	16	0
		Expected Count	14	2
Body Condition	Engorged	Observed Count	21	26
		Expected Count	29	18
	Non-engorged	Observed Count	20	0
		Expected Count	12	8
Life-stage	Adult	Observed Count	34	5
		Expected Count	24	15
	Nymph	Observed Count	7	21
		Expected Count	17	11

There was a significant association between body condition (engorged vs. non-engorged) of the tick and the response to the UG secretion (chi-square, χ^2=13.0.16, df=1, p=0.000). All non-engorged ticks left the square of oil, whereas 45% of engorged ticks remained within it for the duration of the entire experiment (Table 5.2). Likewise, all non-engorged ticks left the square of canola oil (chi-square, χ^2=18.080, df=1, p=0.000; Table 5.3). Significantly more adults left the square of UG secretion (82%) and canola oil (75%) than nymphs (50%; chi-square, χ^2=7.781, df=1, p=0.005 and χ^2=26.536, df=1, p=0.000 respectively) (Tables 5.2 and 5.3).

5.4 Discussion

In this experiment, non-engorged ticks (regardless of sex) moved from the square of oil within seconds of being placed within it. In contrast, 45% (UG secretion) and 55% (canola oil) of engorged individuals remained within the square of oil. This result supports the natural behaviour of ticks, as engorged individuals are most often attached to the host's body and locomotion is restricted (Wall & Shearer, 2001). In contrast, non-engorged individuals are unrestricted in their movements and therefore may disperse from unpleasant abiotic factors, such as large

amounts of UG secretion in/on the feathers of birds, in the search for a more optimal environment (Needham & Teel, 1991).

I. anatis is a parasite of a nidicolous host – one that remains within the nest for a long period after hatch (Starck & Ricklefs, 1998) – thus engorged females do not need to 'seek out' a host as they are most commonly in the presence of one. Unlike the male which has an innate sense to find a receptive female and is therefore active a lot of the time, engorged females and nymphs live a sedentary lifestyle as they do not have to move far to locate a host or a sexually active male (Heath, 2010). Therefore, the above results may reflect the degree of activity within this species rather than their sensitivity towards the UG secretion.

In some instances heavily engorged individuals appeared to have trouble with the oil as it was too slippery for them to pull themselves through. This limitation may have been the reason why some engorged individuals did not leave the squares of oil within the five minute time period. Alternatively the movement of legs could have been impeded by the large body mass each engorged individual had to support. A better approach for this experiment would have been to create a choice test chamber – for example a Petri dish with two divisions and a small area in the middle for the tick. The small area would have openings to allow the tick to choose between the two sides of the dish – one containing the UG secretion and the other containing nothing.

The sex of the tick had an influence on its behaviour towards the UG secretion. As well as all males leaving the square of UG secretion, 70% of engorged females left the square of UG secretion, while only 50% of the experimental nymph population displayed the same behaviour. All experimental nymphs were engorged and were a lot smaller than the females although their body mass to leg length ratio was similar. These results suggest that engorged female ticks dislike the UG secretion more than nymphs, however, this should be interpreted with caution as other variables (such as body size) may have contributed to this outcome. Male tick behaviour is to

search for receptive females (Andrews, 1982). Males may have simply left the square of oil as they are more active than the other stages, in the sense that finding receptive females requires their active movement around the host.

In order to attract males for reproduction, feeding females (engorged) produce sex pheromones (Oliver, 1989). Pine oil has served as a successful repellent of Douglas-fir beetles (*Dendroctonus pseudotsugae*), mountain pine beetles (*D. ponderosa*), and spruce beetles (*D. rufipennis*) toward the attack of Douglas-fir (*Pseudotsuga menziesii*), lodgepole pine (*Pinus contorta*), and spruce (*Picea glauca*) (Nijholt, McMullen, & Safranyik, 1981; Borden, 1989). The pine oil suppressed detection of the host tree pheromone (which attracts the beetles), thus the beetles could not locate their host trees. In this respect, it may be possible that the UG secretion has a similar effect on the sex pheromones of adult female ticks. If sex pheromone detection was suppressed by the presence of UG secretion, males would not be able to locate feeding females and copulation would not occur. Therefore feeding females may re-locate themselves when an encounter with UG secretion occurs and this may be why the engorged females removed themselves from the square of oil more than the nymphs.

In the majority of ixodid tick species the rapid engorgement phase of adult females does not occur until they have copulated with a male (Oliver, 1989). After they are fully engorged (approximately 48 hours post copulation) the female drops off the host and egg laying occurs (Oliver, 1989). In the current experiment 30% of females did not leave the square of UG secretion. Copulation had possibly occurred already and therefore the presence of the UG secretion was not a threat – attracting a male was no longer a concern. Nymphs do not need to copulate before they move into the next stage in their lifecycle, therefore their reaction to the oil would not have been governed by a need to copulate. Consequently whether they remained within the square of UG secretion or whether they left the area did not matter. The reaction of the nymphs towards the UG secretion treatment provides evidence for this hypothesis as 50% left and 50% remained within the square.

Ticks possess a pair of spiracles (stigmata) located lateral to the 4th pair of legs (Sonenshine, 1991). These structures are essential in the tick's longevity and its ability to live for long periods off the host (Sonenshine, 1991; Wall & Shearer, 1991). As described in section 5.1.1 many strategies are employed by birds to combat ectoparasite infestation, including dusting, which has been proposed to block the spiracles leading to asphyxiation (Moyer & Clayton, 2004). To assume that ticks left the square of UG secretion due to a threat of spiracle blockage is hard to accept, as this experiment did not accurately replicate conditions on the host. Furthermore, it is likely that the amount of oil used in the experiment was greater than that normally found in the plumage of birds. Future studies should investigate the UG secretion as a mechanism to combat ectoparasites by using the birds themselves. In this way it would be the bird's use of secretion that is examined and not the tick's behaviour towards aversive situations, as self-awareness is hard presuppose in invertebrates (Mather, 2001).

Overall, more ticks left the square of UG secretion (69%) than remained within it (31%), a result that also occurred in the canola oil treatment (61% and 39% respectively) (Figure 5.9). From this result we can hypothesize that the tick species *I.anatis* may dislike oily substances. However, the reasons for this can still be debated. It is possible that birds could use UG secretion as a means of controlling ticks. The UG secretion may promote the detachment of ticks from the avian host at times that are inadequate to the tick, such as active host periods, and thus re-infection with these individuals within the avian population would be reduced. Although this would be hard to test on Ponui Island due to the sheer numbers of *I.anatis*, this hypothesis could be examined in future experiments in the laboratory (where *I.anatis* numbers could be accurately controlled) and/ or at field locations where ticks are less dense.

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Chapter 6: General Discussion.

The uropygial gland (UG) has perplexed ornithologists for many years. It is specific to birds and is not found in any other animal group. It is an integumentary gland and is located dorsally and medially in the synsacrocaudal region of the bird's body (Jacob & Ziswiler, 1982; Martín-Vivaldi *et al*. 2009; Harem *et al.* 2010). There is a lack of information regarding the structure of the UG, with anatomical studies encompassing only 16% of bird species while only 0.2% of birds UGs have been investigated histologically. Because of the large gaps in knowledge of UG structure, its function has been debated for many years. There are four main hypotheses in the literature regarding the function of the UG: 1) the feather maintenance hypothesis, 2) the water-proofing hypothesis, 3) the intraspecific communication/health hypothesis, and 4) the defence against pathogens and ectoparasites hypothesis (Chapter 1; Martín-Vivaldi *et al.* 2009; Salibian, & Montalti, 2009).

The UG is analogous to the mammalian sebaceous gland (Elder, 1954; Bhattacharyya, 1972) as both have a holocrine arrangement (Jacob & Ziswiler, 1982). In the 0.2% of birds that have had their UGs investigated histologically (Lucas & Stettenheim, 1972; Jacob & Ziswiler, 1982; Sawad, 2006; Harem *et al.* 2010; Sadoon, 2011) each study utilises the same terminology for cellular topography of the follicles of the gland: germinative cells, intermediate cells, secretory cells, and degenerative cells. The 'primary cavity' has been used in these studies too; however, 'cavity' refers to an empty space (Collins Student Dictionary, 2006), which is not true of the UG as these spaces are full of secretion. Therefore, in this thesis we have referred to these areas as 'primary sinuses'.

The main aim of this study was to gain a clear understanding of the structure of the UG in endemic New Zealand birds with consideration of its overall function. New Zealand and its avifauna became isolated from the rest of the world approximately 70 million years ago (Heather & Robertson, 1996). The kiwi (*Apteryx* spp) lineage was an early

offshoot from the primitive ratites and as a result they are some of the oldest endemic members of the New Zealand avifauna (Heather & Robertson, 1996). Likewise hihi and saddleback are endemic to New Zealand and are ancient passerines. This long isolation provided me with an opportunity to gain insight into some of the oldest UGs within two avian groups and to examine any similarities and/or differences.

This study has exposed aspects of the kiwi UG not seen in any other bird. Externally the UG was bilobar, but internally each lobe consisted of four primary sinuses, making a total of eight large storage areas. Each sinus was associated with its own primary duct, thereby providing eight openings in the papilla. Within the kiwi gland, interfollicular septa were also present and caused follicles to be grouped into lobules. The control of UG secretion expulsion is still a large unanswered question in all species of birds. The use of Van Gieson staining revealed tendon-like structures underlying the UG in kiwi. These structures connected the underlying striated coccygeal muscle to the capsule of the UG and could therefore serve a purpose in the control of secretion expulsion. When comparing the cellular composition of the gland between sexes it was found that male kiwi possess more cells in the UG follicle epithelial layer compared to females; thus, male kiwi may produce more secretion than female kiwi.

This study was the first to examine pathological conditions of the UG. The UGs of one brown kiwi (*Apteryx mantelli*), two great spotted kiwi (*A. haastii*) and one Haast tokoeka (*A. australis*) exhibited pathological changes. They indicated that systemic illness may affect the UG, as two cases of UG atrophy, one case of pustular dermatitis, and one case of UG adenitis were discovered in sick kiwi. Pustular dermatitis may be an early case of avian pox infection, which has only recently been reported in kiwi (Ha *et al.* 2011).

Because kiwi are some of the oldest members of the avian fauna, their UG structure could be indicative of the original form of the gland. Within the palaeognathes, all other ratites lack the UG in their adult form except species of Tinamiformes. Tinamous are the only other similar sized palaeognathae related to the kiwi (Johnston, 1988).

Jacob and Hoerschelmann (1985) utilised the UG secretion of kiwi and tinamous in chemotaxonomy and discovered the exclusive occurrence of alkane-2,3-diols. Therefore, tinamous would make excellent candidates to compare the structure of their UGs with that of kiwi, as they both possess similar characteristics in their secretion. The lack of the UG in adults of the other ratite species – which are much taller than both kiwi and tinamous – gives a hint that the UG in these last two species may provide benefits to their ground dwelling existence. Comparisons of the UG between the two ratite groups would complete the information known about UGs of the palaeognathae lineage.

The UG of the New Zealand passerines all possessed the wart-like papilla typical of other members of the order Passeriformes (Jacob & Ziswiler, 1982). Bellbirds possessed the largest gland out of the four species; however, in comparison to passerine species examined by Jacob and Ziswiler (1982), the four New Zealand species grouped together with similar sized glands. Externally, all four species' UGs were similar, although the histological organisation was not. Hihi, tui, and saddleback each possessed three primary sinuses while the bellbird had four. The papilla of bellbirds also contained much more connective tissue compared to the more delicate structures of the other three species. Implications could arise from this in terms of the amount of secretion that could be stored in the bellbird UG. Consequently, the bellbird may obtain less secretion from the gland during preening bouts and therefore their UG may be larger compared to the other three species to accommodate more storage of secretion.

It is interesting that, similar to the starling (Sadoon, 2011), the UG's of hihi, bellbirds, and tui were found to lie across the base of the tail. In comparison to the kiwi, which has a tendon-like structure speculated to control secretion expulsion, the position of the UG in these species may allow for secretion expulsion through the movement of the tail (Jacob & Ziswiler, 1982). Saddleback UGs, however, did not lie directly over the tail, so control of secretion expulsion in this species is still uncertain.

This investigation has shown that although the UG has a general morphological pattern, each species has its own specific differences in the internal structure, since in each passerine studied, the UG expressed dissimilarities. Future investigations could examine the UGs of kokako (*Callaeas cinerea*) and members of the Australian honeyeaters which would aid in explaining the features discovered in this study, to determine whether they are true representations of individual species or occur in other genera within each family. This information could provide further support for their phylogenetic relationship.

Sexual dimorphism within the hihi enticed me into investigating whether or not there were sexual differences within the structure of their UG. In addition, I investigated the effects of season on the histology of the gland. No significant effects of sex were discovered on the cellular characteristics of the UG, and it was concluded that there was no dimorphism between UG characteristics in this species. With the small sample size and the limitations of a Bonferroni corrected p value, there was no significant difference in season, although this should be investigated further with larger samples as a strong tendency towards a larger gland in the winter was detected.

The more we know about the structure of the UG, the closer we come to understanding its function within birds. This study has unveiled for the first time characteristics not seen in other species and has added to the scarce information on the structure of the UG. It has, however, also raised further questions regarding the gland's function. This gland is still enigmatic and much more research is required in order to reveal its true function in each species.

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