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SKIN AS A TOXIN STORAGE ORGAN IN THE ENDEMIC NEW GUINEAN GENUS *PITOHUI*

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ABSTRACT.—Several bird species in the endemic New Guinean genus *Pitohui* contain potent defensive toxins of the batrachotoxin family of steroidal alkaloid neurotoxins. We surveyed toxin concentrations in various tissues of Hooded Pitohui (*Pitohui dichrous*) using radioligand binding assays. The highest concentrations were found in the skin and feathers, on the outside of the birds, where predators or parasites are likely to encounter toxins. Significant levels of toxins also were found in skeletal muscle, heart, and liver. Muscle and liver would normally be poisoned by batrachotoxins; thus, Hooded Pitohuis must be insensitive to the toxins. The presence of toxins in internal organs further argues against the hypothesis that Hooded Pitohuis merely apply toxins topically to skin and feathers. Finally, we used scanning and transmission electron microscopy to examine skin and feathers for unusual morphological or histological adaptations for storing and secreting toxins. The skin of Hooded Pitohuis appears to have typical dermal and epidermal morphology, and we speculate on possible ways in which this species may sequester and secrete toxins using typical avian skin structural features, unique among vertebrates. *Received 10 November 2008, accepted 16 January 2009.*

Key words: batrachotoxin, chemical defense, feathers, Hooded Pitohui, *Pitohui dichrous,* skin.

La Piel como Órgano de Almacenamiento de Toxinas en el Género Endémico de Nueva Guinea *Pitohui*

RESUMEN.—Muchas especies de aves del género endémico de Nueva Guinea *Pitohui* contienen potentes toxinas defensivas, neurotoxinas alcaloides esferoidales de la familia de las batracotoxinas. Estudiamos las concentraciones de toxinas en varios tejidos de *Pitohui dichrous* empleando ensayos de unión de radioligandos. Las mayores concentraciones fueron encontradas en la piel y las plumas, en la parte externa de las aves, donde los depredadores o los parásitos tienen más probabilidad de encontrarse con las toxinas. También se encontraron niveles significativos de toxinas en el músculo esquelético, corazón e hígado. El músculo y el hígado serían normalmente intoxicados por las batracotoxinas; por ende, *P. dichrous* no debe ser sensible a éstas. La presencia de toxinas en los órganos internos es un argumento adicional en contra de la hipótesis de que *P. dichrous* simplemente aplica las toxinas tópicamente a la piel y las plumas. Finalmente, utilizamos microscopía electrónica de barrido y de transmisión para examinar la piel y las plumas en búsqueda de adaptaciones morfológicas o histológicas inusuales para almacenar y secretar toxinas. La piel de *P. dichrous* parece tener una morfología dérmica y epidérmica típica. Especulamos sobre los posibles modos en los cuales la especie puede secuestrar y secretar toxinas usando las características estructurales típicas de la piel aviar, únicas entre los vertebrados.

POTENT NEUROTOXIC ALKALOIDS have been isolated and identified from the feathers and skins of six New Guinean bird species, the Blue-capped Ifrita (*Ifrita kowaldi*) and five species in the genus *Pitohui* (Dumbacher et al. 1992, 2000). The toxins belong to the batrachotoxin family of compounds (BTXs) that bind and activate sodium channels in nerve and muscle membranes (Albuquerque et al. 1971). On a molecular-weight basis, BTXs are among the most toxic natural substances known—more toxic than curare or strychnine. It is not commonly reported that birds carry such toxins (for a review, see Dumbacher and Pruett-Jones 1996), and we sought to further describe and understand the toxicity of pitohuis by carefully surveying key tissues from the Hooded Pitohui (*Pitohui dichrous*). We use these data to address or further develop several hypotheses related to the evolution and function of toxins in pitohuis.

Before considering whether the toxins may be used for chemical defense, we must first ask what are the potential target enemies of defensive chemicals? Birds have multiple enemies that defensive

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chemicals could deter, including ectoparasites (e.g., lice, mites, ticks, and hippoboscid flies) and predators (e.g., snakes, hawks, arboreal mammals, and even humans). Experimental evidence suggests that BTXs can both kill and repel lice (Dumbacher 1999), and field surveys suggest that pitohuis have lower incidences of some ectoparasites than sympatric bird species (Mouritsen and Madsen 1994). In predators such as the Brown Tree Snake (*Boiga irregularis*) and Green Tree Python (*Morelia viridis*), pitohui toxins irritate buccal membranes (J. Dumbacher unpubl. data). In humans, merely handling the birds can irritate nasal and buccal tissues, cause sneezing and allergic-like symptoms (Salvadori 1881). Batrachotoxins are known to protect pitohuis from local people who hunt and eat other passerines of similar size (Salvadori 1881; Majnep and Bulmer 1977; Kocher-Schmid 1991, 1993). Batrachotoxins poison by binding to voltage-gated sodium channels in cell membranes of central nervous and muscular systems. They lock the channels open, allowing a flood of sodium ions that depolarizes the cell membrane. Batrachotoxins are known to be toxic in a wide variety of animals, including vertebrates and invertebrates (Daly and Spande 1986, Dwivedy 1988, Soderlund et al. 1989), and could, therefore, provide defense against a wide spectrum of potential natural enemies—from large vertebrate predators to tiny mite ectoparasites.

For BTXs to function defensively, toxins should be located in tissues where enemies will readily encounter the toxins and can react accordingly. For the enemies mentioned, this means the outside of the birds—primarily the skin and feathers. This is where ectoparasites live, feed, and breed, and these are the tissues that predators encounter first. Although avian toxins have been found in other tissues—bone of Common Bronzewing (*Phaps chalcoptera*; Gardner and Bennetts 1956, Main 1981), viscera of Carolina Parakeet (*Conuropsis carolinensis*) and Spur-winged Goose (*Plectropterus gambensis*) (Wilson and Bonaparte 1831, Eisner 1993), and muscle of Eurasian Quail (*Coturnix coturnix*; Kennedy and Grivetti 1980, Lewis et al. 1987)—a predator would have to kill the prey before it could detect and react to poisons. By examining which pitohui tissues contain toxins, we can test whether toxins may be useful for defense and we can postulate which enemies are most likely defended against.

Another important question is how toxins are acquired. Toxins can be acquired from diet (Daly et al. 1994, 1997; Hutchinson et al. 2007), applied topically (Weldon 2004), or produced *de novo*. If applied topically, one would expect toxins to be found primarily externally on skin and feathers and not in internal tissues. If produced *de novo*, a survey of tissues may help locate the organs or organelles that are high in toxins, and these can be further examined to see whether there is evidence for special structures for toxin storage, production, or secretion. Furthermore, species that acquire toxins from their diet often show tremendous variation in toxicity among individuals or among localities, presumably because toxins are more or less available from one locality to another, or for one individual compared with another (Brower et al. 1982, Daly et al. 1992). Thus, variation in toxicity among individuals or localities may suggest that pitohuis acquire toxins from their environment.

A fourth question asks whether pitohuis are insensitive to high levels of their own toxins. Because BTXs target voltage-gated sodium channels in nerve and muscle membranes, high levels of BTXs in muscle would indicate that pitohuis have reduced BTX sensitivity (Daly et al. 1980).

We surveyed toxin distributions from 15 wild-caught Hooded Pitohuis from four collecting localities. We compared toxins among several tissues, including skin, feathers, skeletal muscle, and internal organs, to determine which tissues contained toxins and their relative concentrations of toxins. Although the present study is primarily descriptive, the results have clear significance for several of the questions mentioned above.

Although toxin surveys of pitohuis have been reported before (Dumbacher et al. 1992, 2000), these studies each had limitations. The first report of pitohui toxins (Dumbacher et al. 1992) surveyed only a few individuals and, thus, was unable to address variation among tissues within individuals or variation among individuals. Field experiences suggested that significant variation exists and may affect the biological relevance of pitohui toxicity. Furthermore, that first report (Dumbacher et al. 1992) recognized only one BTX compound, homobatrachotoxin. Later, several other BTX compounds were found in the Blue-capped Ifrita and pitohuis, and relative amounts of these compounds were estimated for various tissues (Dumbacher et al. 2000). Because the toxicity of these other BTX compounds remains unknown, the toxicity of various bird tissues cannot be inferred from those data (Dumbacher et al. 2000).

In the present study, we used a radioligand binding assay that directly measured the binding of toxins to voltage-gated sodium channels. Rather than looking primarily at the chemical analyses (Dumbacher et al. 2000), we focused on the biological function of the chemicals. This provides a proxy measure for the "toxicity" of samples and has direct ecological relevance. Additionally, by collecting multiple individuals from each locality and multiple tissues from each individual, we were able to assess variation at multiple levels: among localities, among individuals, among tissues, and even among the body locations of some tissues. This had never been done before for any bird species and is relatively rare in toxin research, even among well-studied toxic animals.

Finally, we used scanning and transmission electron microscopy to investigate the tissues that contained the highest levels of BTXs. We examined these tissues for specialized structures that might be involved with toxin production, storage, or secretion.

Methods

Field work and sample collection.—For the tissue toxin comparisons, birds were collected and sacrificed from a total of four localities (Baitabag, Balbe, Kaironk, and Nokopo villages; Table 1). A voucher half skin (dried flat with one wing and leg) and skeleton were prepared for each bird. The entire remaining half skin and feathers were prepared for toxin analyses. Feathers were plucked from the head, back, breast, wings, and legs of each half skin, and all feathers of each type were placed in separate plastic Ziplock bags. Skin, breast muscle, leg muscle, heart and liver (combined), and stomachs and their contents were separated and stored in 10 mL of absolute ethanol in separate glass vials with Teflon-lined lids. Tools were wiped clean after each sample was prepared, and hands were washed after preparing each individual bird to prevent transferring toxins. For two apparently highly toxic birds from Nokopo (evidenced by the fact that merely skinning them irritated our eyes and nasal passages), skin was collected from head, back, breast, and legs and stored separately for comparison. Skin samples for

Table 1. Elevation (meters above sea level), habitat, number of Hooded Pitohuis collected (*n*), latitude and longitude (in decimal minutes), and collection dates for each collection locality in the present study. All localities are in Madang Province, Papua New Guinea.

Locality	Elevation (m)	Habitat	*n*	Latitude, longitude	Collection dates
Baitabag Village, near Madang	100	Secondary forest	2	5°6.6′S, 145°47′E	23 September 1993
Nokopo Village, Finisterre Mountains	2,000	Village garden	3	5°55.8′S, 146°36′E	7–17 October 1993
Balbe Village, Adelbert Mountains	1,000	Secondary forest	5	4°56.4′S, 145°36′E	1–6 December 1993
Kaironk Valley, Central Ranges	1,850	Village garden	5	5°5.23′S, 144°28.4′E	4–8 January 1994

electron microscopy work were prepared in the field by making thin slices and storing them in glass vials in modified Karnovsky fixative (Karnovsky 1965, Ghadially et al. 1992). Batrachotoxins are stable dried or in ethanol, but we took extra precautions to keep samples in cool dark places while in the field and refrigerated them as soon as they were brought back to the field station. In our analyses, there was no evidence that toxins degraded in storage.

Sample preparation in the laboratory.—Toxin extractions were performed at the Laboratory of Bioorganic Chemistry, National Institutes of Health. Tissues, vials, and ethanol were weighed on an analytical balance, and the weight of the empty vial plus the weight of ethanol were subtracted to give the wet weight of the tissue sample. The sample with ethanol was placed in a glass mortar, and tissues were cut into fine pieces and ground with a pestle. Macerated tissue was filtered under vacuum, the filtrate was collected, and the tissue was washed three times with 100 µL methanol. The tissue was dried overnight at room temperature and weighed with an analytical balance to give the dried weight of the tissue sample. The average amount of tissue extracted was then determined. The combined methanol and ethanol filtrates were concentrated to dryness under vacuum with a rotary evaporator. The residue (BTXs are not volatile) was resuspended in 2 mL methanol per gram of wet tissue.

Toxins were extracted from small quantities of feathers as follows. Approximately 100 µg of feathers (~15 contour feathers) were weighed on an analytical balance. A tiny hole was drilled in a 0.5-mL plastic microcentrifuge tube using a 28-gauge needle, and the feathers were loosely packed into the tube. Feathers were soaked in 2 mL of ethanol per gram of feathers and allowed to sit for 5 min, which allowed the toxins to dissolve in the ethanol. The 0.5-mL centrifuge tube with feathers and ethanol was then placed inside a 2-mL centrifuge tube and spun in a bench-top microcentrifuge at 14,000 rpm for 5 min to drain the ethanol away from the feathers. This washing procedure was repeated three times, and ethanol washes were combined and dried under a nitrogen stream. The residue was resuspended in 2 mL methanol per gram of feathers.

Toxin assay techniques.—We used an established BTX binding assay, which is a competitive radioligand binding assay that provides an accurate measure of the toxicity of each sample (Creveling and Daly 1990). Toxicity is expressed in units of BTX equivalents per gram of tissue (µg BTX eq./g tissue) such that 1 µg BTX equivalent indicates the presence of toxins in an extract having the same binding effect as 1 µg BTX. Batrachotoxin equivalents in pitohuis represent contributions from a number of BTX compounds (Dumbacher et al. 2000, 2004), but the toxicity of many of these individual compounds remains unknown. Batrachotoxin equivalents derived from the binding assays therefore provide an assessment of the overall alkaloid activity of each pitohui extract. Because the assay determined the combined bioactivity of the extracts, we think that this is an ecologically relevant quantification of toxicity and is superior to any previously reported avian toxicity studies.

Batrachotoxin binding assays were conducted essentially as described by Creveling and Daly (1990), but using guinea pig synaptoneurosomes instead of rat synaptosomes. Guinea pig cortical synaptoneurosomes were obtained as described by Hollingsworth et al. (1985). Details about supplies, buffers, and exact lab procedures are described in detail by Dumbacher (1997).

We used analysis of variance (ANOVA) to analyze toxin concentrations using the general linear models (GLM) procedure in SAS (SAS Institute, Cary, North Carolina) and XLSTAT (Addinsoft, New York). Sampling design was often unbalanced, requiring type III and type IV calculations of sums of squares. The sources of unbalanced samples were primarily differing numbers of BTX binding experiments that produced toxicities within a range that could be interpolated. Field sampling differences affected the sampling balance in only a couple of cases; for example, birds from Nokopo were highly toxic, and this allowed us to test for differences in toxicity among body locations within a single tissue type (i.e., skin from head, back, abdomen, and legs), but this also produced several different estimates of skin toxicity for birds from Nokopo that had to be statistically pooled before comparing bird skin from other areas. Sampling allowed us to test for significant effects at multiple levels, including variation among tissue types (four classes: feathers, heart and liver, muscle, and skin), variation among individuals collected ($n = 15$), variation among replicate extracts, and variation among field collecting localities ($n = 4$). Error mean squares were estimated using variation among replicate BTX binding experiments and variation among the three replicate test tubes of each BTX binding experiment. Residuals were examined, and their distribution did not differ from a normal distribution.

Microscopy.—For transmission electron microscopy, skin samples from the back and breast regions were fixed in modified Karnovsky fixative (2% glutaraldehyde, 2% paraformaldehyde with 0.06% calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.4), following Hou et al. (1991), washed extensively in 0.1 M sodium cacodylate buffer, and minced into 1-mm pieces using a razor blade. These samples were then postfixed in 1% OsO_4 for an hour. Following osmication, samples were washed in buffer, routinely dehydrated in a graded series of ethanol solutions, changed briefly to propylene oxide, before infiltrating with and embedding in a low-viscocity Epon epoxy mixture (Hexion, Houston, Texas). Silver-gray sections were cut on an ultramicrotome using a diamond

TABLE 2. Results of ANOVA comparing toxin concentrations in skin extracts. Extracts were made from back, breast, head, and leg of two Hooded Pitohuis from Nokopo. Variance is partitioned among a number of effects, including skin location, individual tested, a skin location*individual interaction, and tube (number of replicate tubes in batrachotoxin binding assays.) Type III mean squares (MS) were used to minimize the effect of unbalanced design (primarily introduced from repeated toxin assays.)

Source	df	MS	*F*	*P*<
Skin location	3	3,482.067	10.46	0.0426
Individual	1	1,096.026	10.70	0.0048
Location*individual	3	332.799	3.25	0.0495
Error	16	102.413		

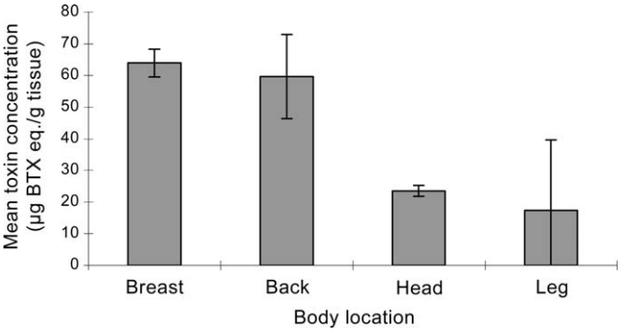

FIG. 1. Mean toxin concentration (µg BTX eq./g tissue; see text) of skin of Hooded Pitohuis, by body location. Error bars depict SD.

knife, picked up on copper grids (300 mesh size), and double stained with uranyl acetate and lead citrate. These were examined under a Zeiss 10 electron microscope operated at 80 KV.

For scanning electron microscopy of skin and feathers, the outer layers of skin (stratum corneum) were sampled from birds using D-SQUAME tapes (CuDerm, Dallas, Texas). Individual feathers were also separately collected on D-SQUAME tapes. These tapes were sputter-coated with gold and examined and photographed under a scanning electron microscope.

RESULTS

Variation with respect to tissue location within individual Hooded Pitohuis.—In two Hooded Pitohuis from Nokopo, skin from back, breast, head, and legs was stored and assayed separately to test for differences in toxin concentration in skin among body locations. Because of the small quantities of skin available from each bird, this was attempted only with two individuals that were obviously highly toxic, given the reactions we experienced while handling the birds in the field. We detected a difference in skin toxin concentrations taken from different body locations (ANOVA type III MS, $F = 10.46$, df = 3 and 3, $P < 0.0427$; Table 2). Results are presented as mean (± SD) µg BTX eq./g tissue. Skin collected from the breast (63.9 ± 17.1) and back (59.6 ± 11.3) was more than twice as toxic as skin from the head (23.5 ± 3.9) and leg (17.3 ± 17.6) in pairwise *t*-tests controlled for multiple comparisons ($P < 0.05$; Fig. 1). Among feathers in the same two individuals, back feathers (43.0 ± 34.3) and breast feathers (38.9 ± 19.2) were more toxic than feathers collected from head, leg, or wing (27.2 ± 15.5, 15.3 ± 7.3, and 10.3 ± 4.3, respectively), but differences were not statistically significant. There were no significant differences between skeletal muscle collected from breast and leg (2.9 ± 1.7 and 3.2 ± 3.1, respectively).

Variation among tissues within an individual.—Toxin concentrations differed significantly among skin, feathers, heart and liver, and skeletal muscle (one-way ANOVA, $F = 29.682$, df = 3 and 309, $P < 0.0001$). Overall, skin and feathers were most toxic (26.7 ± 20.1 and 15.5 ± 17.2, respectively), followed by heart and liver (10.4 ± 7.6), and skeletal muscle was least toxic (2.9 ± 1.7 µg; Fig. 2). Within most individuals, skin was most toxic; however, two Hooded Pitohuis from Nokopo contained higher toxin concentrations in their feathers than in their skin. Furthermore, the three birds with the highest skin toxin levels, all collected from Balbe Village, had among the lowest feather toxin concentrations. These findings suggested a tissue*locality interaction. Thus, the effects of locality and tissue*locality interactions were included in subsequent ANOVA designs for examining tissue variation. Subsequent tests revealed strong direct effects of locality on toxicity (ANOVA, $F = 28.819$, df = 3 and 297, $P < 0.0001$) and a strong tissue*locality interaction (ANOVA, $F = 12.38$, df = 9 and 297, $P < 0.0001$; Table 3). This suggests that not only do toxin levels differ among localities, but the distribution of toxins among tissues may also differ among localities.

Transmission electron microscopy results.—Pitohui epidermis was composed of four or five cell layers of sebokeratinocytes, which is typical for avian body epidermis (Menon et al. 1986; Figs. 3A–C). The basal cells (stratum basale) resting on the basement membrane were characterized by large nuclei and cytosol with a few keratin filaments, rough endoplasmic reticulum, mitochondria, and Golgi complexes. Suprabasal cells exhibited the beginning of lipogenesis, as exemplified by free lipid droplets (Fig. 3B). Above this layer is the stratum transitivum, clearly definable by its increased lipid contents, both in the form of electron lucent lipids and as the pleiomorphic multigranular bodies analogous to the epidermal lamellar bodies of mammals (Elias et al. 1987, Menon et al. 1989). In some samples, free lipid droplets are more numerous (Fig. 3C), whereas in others the multigranular bodies predominate (Fig. 3B). This variation may be attributable to the samples coming from different epidermal proliferation units or to facultative changes in the permeability barrier of individual birds. We observed prominent Golgi complexes in the basal cells, with some of the Golgi compartments being strikingly electron-dense because of high staining with osmium tetroxide (Fig. 3B, arrows).

An abrupt transition into the stratum corneum is marked by corneocytes with a large core of lipid resulting from fusion of individual lipid drops, dissolution of cellular organelles, and a thickened cornified envelope. The stratum corneum of Hooded Pitohuis was composed of as many as 40 cell layers. This thickened stratum corneum is characteristic of avian epidermis and not unique to pitohuis; birds generally have more layers than terrestrial mammals, which typically have 7–18 layers.

Scanning electron microscopy results.—In the corneocytes harvested by a D-SQUAME strip, the examined surface is the undersurface of corneocytes as opposed to the outer surface (Fig. 4A). The corneocytes appear as an undulating surface of irregularly shaped squames, but with a very smooth surface. Embedded within this

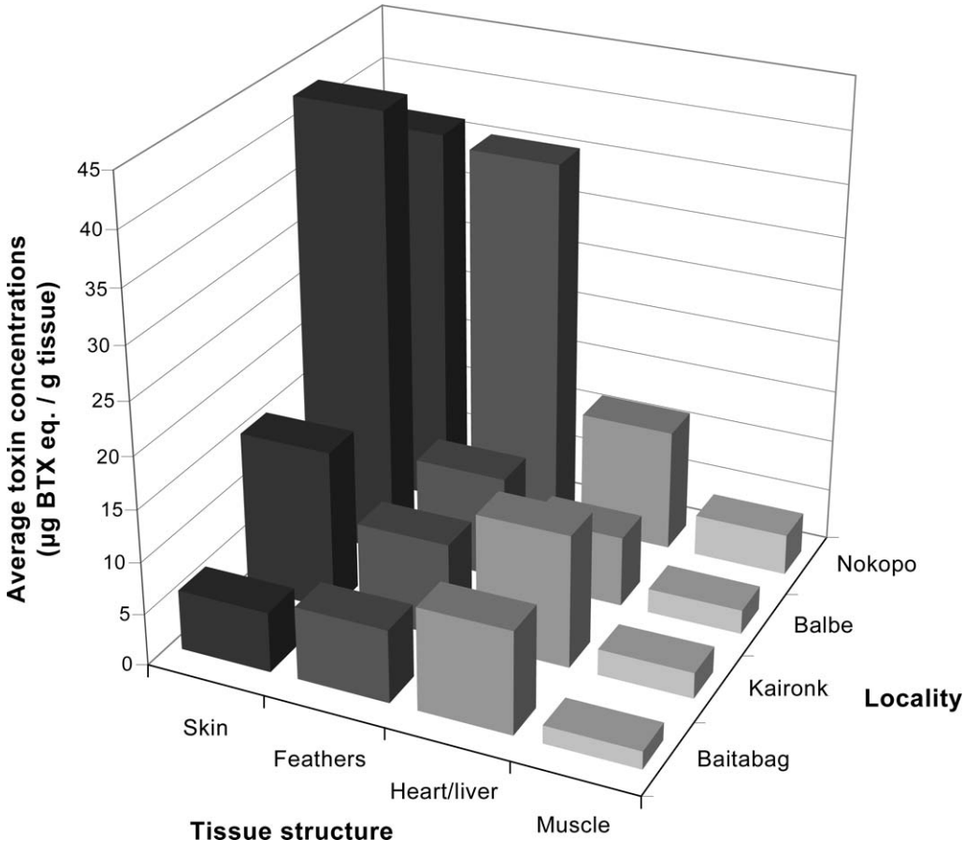

Fig. 2. Average toxin concentrations of tissues of Hooded Pitohuis, by locality and tissue. Note that at Baitabag, feathers were slightly more toxic than skin, whereas at most localities skin toxin concentration exceeded that of feathers.

surface are several clumps of globular structures (Fig. 4A). These are resolved as skin microflora at higher magnification (Fig. 4B). Smooth coating of lipids and attached globular structures that resemble skin microflora are seen on the feathers as well (Fig. 4C, D).

Discussion

Significant differences in toxin concentrations were found among skin samples collected from head, leg, breast, and back, though no statistically significant differences were found among feather or skeletal muscle samples taken from different body locations. The overall higher toxicity of skin may have increased the possibility of detecting differences. Feather extracts exhibited greater variation in toxicity overall, which made it difficult to detect systematic differences in feathers from different body locations. Feather extracts were usually prepared from ≤10 feathers, so variation in feather extracts could be attributable to variation among individual feathers caused by aging and toxin chemical decomposition, wear, recent preening, bacterial degradation, feeding by feather mites, or other causes.

Table 3. Results of ANOVA comparing toxin concentrations among tissues. Extracts were made from feathers, heart and liver, muscle, and skin of 15 Hooded Pitohuis. Type III mean squares (MS) were used to minimize the effect of unbalanced design (primarily introduced from repeated toxin assays.)

Source	df	MS	*F*	*P*<
Tissue	3	4,497.367	32.044	0.0001
Locality	3	2,577.584	18.36	0.0001
Tissue*locality	9	1,738.677	12.39	0.0001
Error	297	140.351		

To offer the reader a perspective on these different BTX levels, a single feather taken from the breast or back of a Hooded Pitohui from Nokopo (~40 µg BTX eq./g feather), if placed on one's tongue, would cause a burning, tingling sensation that would last for several hours or overnight. Merely handing these birds—removing them from nets and preparing specimens—caused us to sneeze, experience watery eyes and runny noses, and generally respond as if we were having allergic reactions. Similar experiences with pitohuis have been reported by other ornithologists (Salvadori 1881) and by anthropologists working in Nokopo (Kocher-Schmid 1991, 1993). For comparison, birds from Baitabag (~5 µg BTX eq./g feather) did not have the same effects; tasting their feathers produced, at most, only a slight tingling sensation,

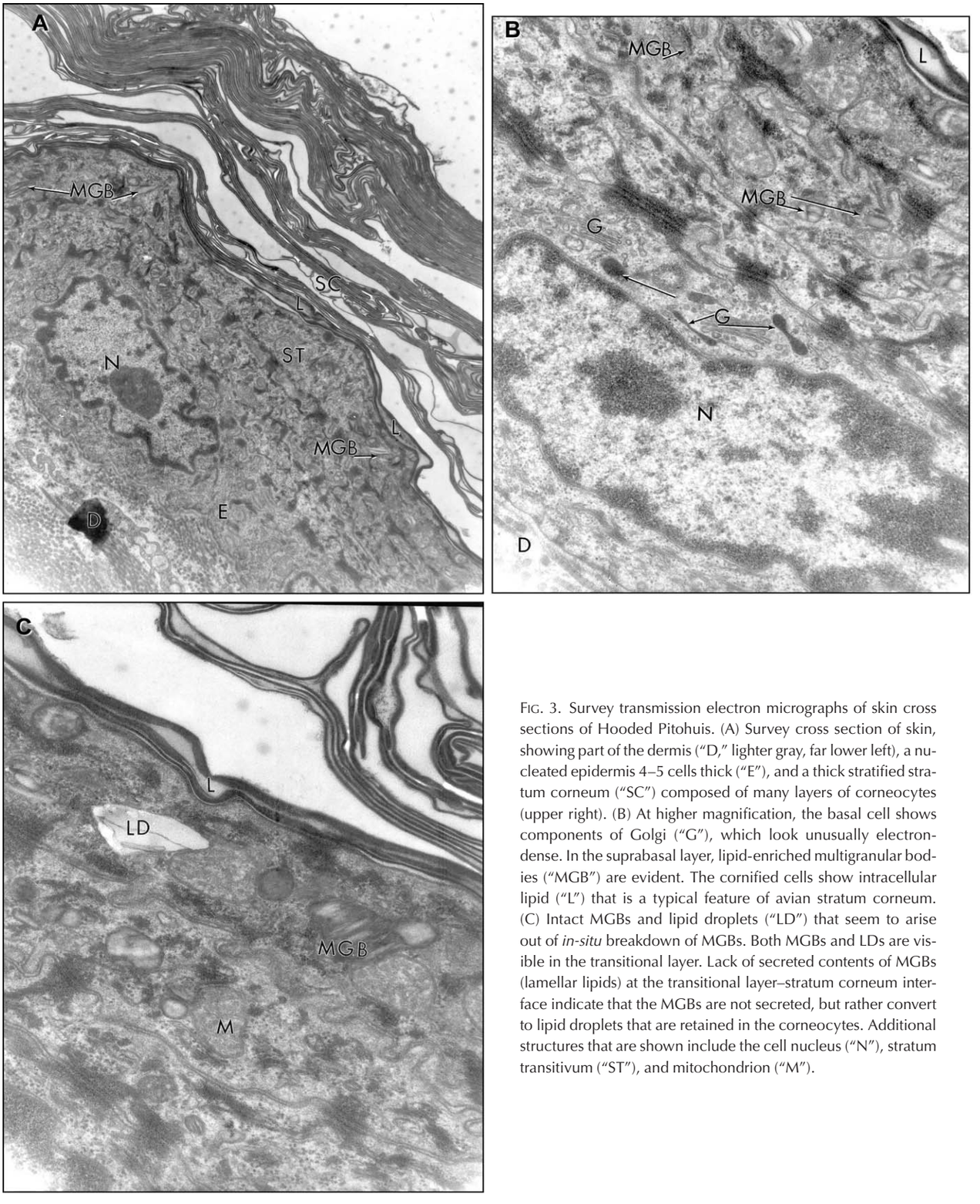

FIG. 3. Survey transmission electron micrographs of skin cross sections of Hooded Pitohuis. (A) Survey cross section of skin, showing part of the dermis ("D," lighter gray, far lower left), a nucleated epidermis 4–5 cells thick ("E"), and a thick stratified stratum corneum ("SC") composed of many layers of corneocytes (upper right). (B) At higher magnification, the basal cell shows components of Golgi ("G"), which look unusually electron-dense. In the suprabasal layer, lipid-enriched multigranular bodies ("MGB") are evident. The cornified cells show intracellular lipid ("L") that is a typical feature of avian stratum corneum. (C) Intact MGBs and lipid droplets ("LD") that seem to arise out of *in-situ* breakdown of MGBs. Both MGBs and LDs are visible in the transitional layer. Lack of secreted contents of MGBs (lamellar lipids) at the transitional layer–stratum corneum interface indicate that the MGBs are not secreted, but rather convert to lipid droplets that are retained in the corneocytes. Additional structures that are shown include the cell nucleus ("N"), stratum transitivum ("ST"), and mitochondrion ("M").

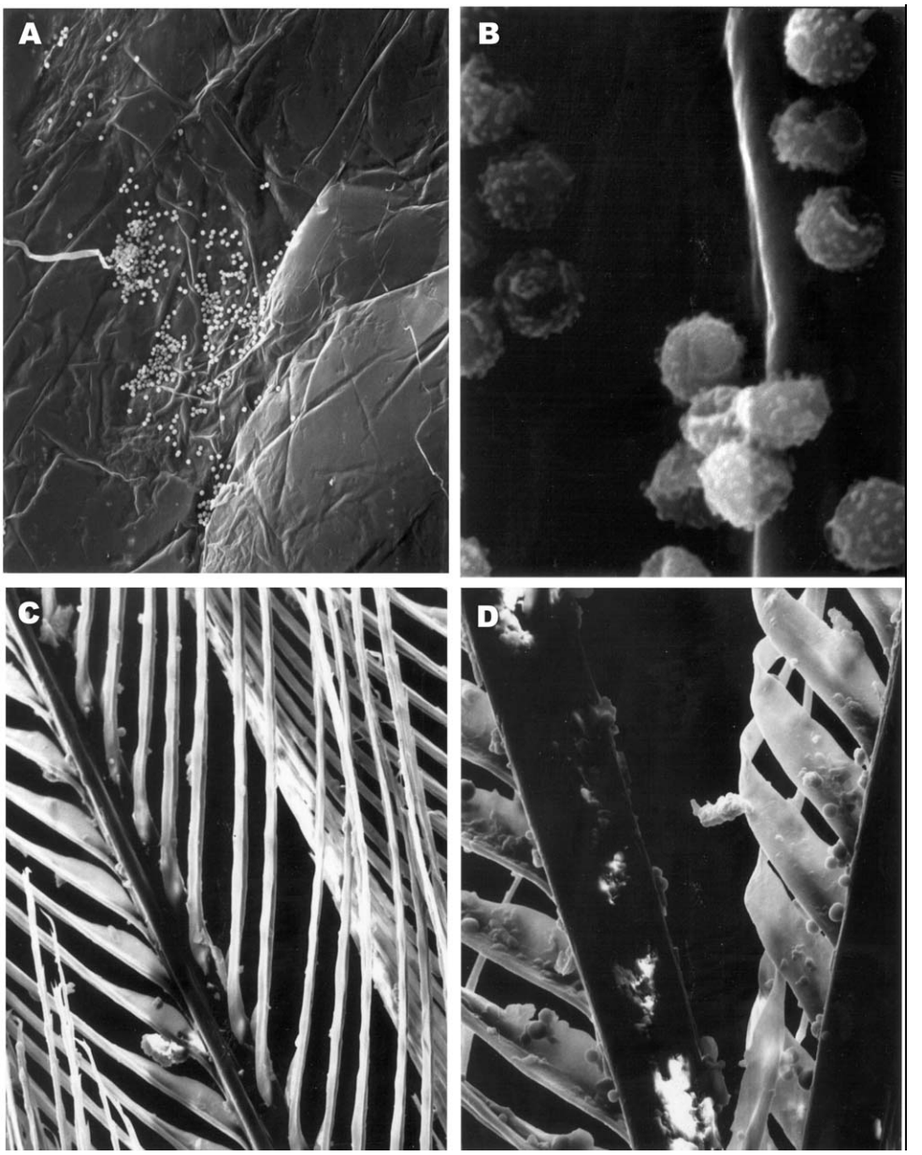

Fig. 4. Scanning electron micrographs of stratum corneum of Hooded Pitohuis, sampled with D-SQUAMES tape in the field. (A) Low magnification shows abundant skin microflora. (B) Higher magnification of the microbial flora. (C and D) Scanning electron micrographs of a feather showing adherent lipids and microflora at different magnifications.

and handling birds never caused sneezing, watery eyes, or running noses.

Relative tissue toxin concentrations were similar to those found in other studies (Dumbacher et al. 1992, 2000); skin had the highest concentrations and feathers the second highest, but internal organs (heart and liver) and skeletal muscle also contained detectable toxin concentrations. In contrast to the results of a previous study (Dumbacher 1994), skeletal muscle had lower toxin concentrations than heart and liver (Fig. 3).

The presence of high concentrations of toxins in external tissues (skin and feathers) is consistent with the hypothesis that the toxins provide a chemical defense for pitohuis. For defense, toxins ideally should be located on the outside of birds, where potential enemies, such as predators and parasites, could readily encounter the poisons and react appropriately.

The presence of toxins in skin and feathers suggests that they would be available for defense against ectoparasites. For example, feathers and skin are the tissues in which mites, fleas, ticks, and lice live, feed, and, in some cases, breed. Evidence suggests that BTXs may both deter ectoparasites and shorten their life span, thus acting as both an insect repellent and an insecticide (Dumbacher 1999). If BTXs also affect blood-feeding

arthropods such as mosquitoes and black flies (Weldon et al. 2006), toxic feathers and skin may also reduce both the direct effects of these insects on fitness and the incidence of insect-vectored parasite infections, such as *Plasmodium*, *Haemoproteus*, or *Leucocytozoan*.

The presence of toxins in feathers and skin suggests that toxins are available for defense against predators as well. Several common New Guinean bird-eating hawks are large enough to depredate pitohui-sized birds, and we expect that they would be sensitive to the toxins. Several common bird-eating snakes, including the Brown Tree Snake and the Green Tree Python, are common in New Guinea, and both species react strongly to naturally occurring toxin levels (J. Dumbacher unpubl. data.) An individual pitohui may survive an attack if predators react quickly enough to the toxins, but pitohui populations may also be protected if predators learn to avoid them. We know that this is the case for human predators, because local hunters know that pitohuis are inedible (Majnep and Bulmer 1977).

The presence of detectable toxins in the skeletal muscle and in the heart and liver clearly shows that pitohuis are insensitive to the toxins. All these tissues contain requisite voltage-gated sodium channels that would be targeted by BTXs, which are among the most toxic natural substances known and have a minimum lethal dose in mice of ~0.04 μg (subcutaneous injection; Myers et al. 1978). On average, 1 g of pitohui muscle contains approximately 3–4 μg of BTX equivalents—enough toxin to paralyze or kill dozens of mice, and certainly enough to affect pitohui muscle tissue if it were sensitive.

We do not yet know how pitohuis have achieved their insensitivity. Frogs of the genus *Phyllobates* that contain BTX have evolved BTX-insensitive sodium channels (Albuquerque et al. 1973, Daly et al. 1980). Other studies have shown that BTX resistance can be attained by single point mutations in the sodium channel gene (Wang et al. 1998, 2000; Wang and Wang 1999). It is possible that pitohuis have likewise evolved a resistant sodium channel. An alternative explanation is that pitohuis carry an additional chemical that affects BTX binding to the sodium channel. Our work with crude muscle extracts suggested that muscle of Hooded Pitohuis may contain additional compounds that reduced BTX binding and sodium flux, but we have not yet purified and identified these compounds.

Finally, we are interested in how pitohuis acquire toxins—whether they manufacture them *de novo*, apply them topically by anointing (Clayton and Vernon 1993, Clayton and Wolfe 1993, Weldon 2004), or sequester them from dietary uptake. Although our study provides no direct test of these hypotheses, the data are consistent with an animal that sequesters toxins from its diet. First, the presence of significant toxin quantities in internal organs—heart, liver, and skeletal muscle—suggests that BTXs are not merely topically applied. Second, toxicity varied tremendously among geographic localities (Fig. 4), which is typical of species that sequester toxins from their diets. Geographic variation in toxicity often reflects the local availability of toxins; for example, Monarch Butterflies (*Danaus plexippus*) are more or less toxic depending on the toxin reserves in their local larval host plant (Brower et al. 1982). Third, a small New Guinean beetle in the genus *Choresine* (family Melyridae) has recently been found to contain BTXs (Dumbacher et al. 2004). Pitohuis consume beetles of that genus (Dumbacher et al. 2004), so it is possible that pitohuis sequester BTXs from them. Fourth, alkaloids are found mostly in plants and a few arthropod groups (Blum 1981), and birds are not known to produce alkaloids or alkaloid-like chemicals. Fifth, in the few cases of toxicity or potential chemical defense in birds, it is believed that the birds sequester toxins from their diet (Dumbacher and Pruett-Jones 1996). Finally, the only other vertebrates known to carry BTXs, frogs in the genus *Phyllobates*, sequester BTXs from their diet (Daly 1995).

In our analyses, we found a striking and unexpected interaction of tissue with locality. The highest toxin concentration was in feathers at one locality (Nokopo), but in skin at the other localities. The causes for this are unknown, but they may be related to the mechanism by which pitohuis take up dietary toxins and transport and incorporate toxins into feathers. Toxin concentrations could be very high in new feathers and decline over time through chemical degradation, washing, wear, and so on. Alternatively, feathers could be virtually nontoxic when first produced and accumulate toxins over time through preening and contact with toxic skin. In either case, these scenarios could explain why the toxin concentrations vary between feathers and skin among localities. Nothing is known about the natural molt schedules of pitohuis, so future studies are needed to investigate how toxins are incorporated into feathers. It is also important to note that because one researcher (J.P.D.) collected all of the field samples and visited each locality only once, any effects of locality and date are confounded in the locality variable.

We examined epidermis for unique morphological features that may be implicated in sequestering, storing, or secreting toxins, but we encountered no such specialized structures, such as glands or ducts, in Hooded Pitohuis that differed from those of other passerines. Our findings are consistent with past work on avian integument that has led to the view that the entire avian epidermis should be considered a holocrine unit (Lucas 1970, Menon et al. 1980, Menon 1984, Menon and Menon 2000). Because of the skin's adaptations as a holocrine unit and as a lipid-rich water barrier, the skin may be pre-adapted or exapted (Brower et al. 1988) for sequestering and storing certain types of compounds, including defensive toxins. The high turnover rate of epidermis, which compensates for the continuous exfoliation of the cells from the stratum corneum, is one potential route of elimination of toxic substances from the body (Teichmann et al. 2005), as is the "storage excretion" via feathers. Although we are not sure whether the toxins in feathers are bound to compounds such as melanin, epidermal cells may be adapted for toxin sequestration and create a surface "depot" of toxins. Regardless of the mechanism, chemical defense is a newly recognized function added to the multifaceted epidermal barrier function. A recent report on such defensive chemical accumulation in nuchal glands of a snake (Hutchinson et al. 2007) is a case in point, though the latter is a truly dermal gland.

Where in the epidermis could the toxin be sequestered? Because BTXs are lipophilic, they may be selectively incorporated in the lipids synthesized by epidermal sebokeratinocytes. This could also be inferred from the osmiophillic Golgi compartments of the basal epidermal cells. It is now known that the multigranular bodies (and lamellar bodies, their orthologues in mammals) bud off the Golgi network. As the epidermal cells differentiate further

and stratify, more of the multigranular bodies are formed, the final fates of which are determined by the xeric stress to which the birds are subjected (Menon et al. 1996). If the permeability barrier needs to be strengthened, the multigranular bodies are secreted as lamellar lipid disks, which unfurl and fuse with each other, forming extensive occlusive lipid bilayers spanning the extracellular spaces in the stratum corneum. As with the mammalian lipid bilayers, avian multigranular bodies also contain a battery of enzymes (Menon et al. 1991) and, in the case of pitohuis, possibly the sequestered toxins. In birds not under xeric stress, the multigranular bodies break down *in situ* into individual lipid droplets, which tend to coalesce with each other and form the core of the lowermost corneocytes where the toxins could be trapped. However, as individual corneocytes move within the stratum corneum toward the skin surface (to replace the outermost cells that are shed), they flatten, and the lipids from the core escape through porosities in the membrane, filling the stratum corneum's extracellular domains with nonlamellar lipids, which could provide a depot for the toxins that are cosecreted with the lipids. Indeed, the lipids of the mammalian stratum corneum are known to provide a depot for topically applied drugs (Teichmann et al. 2005). Previous studies suggested that the epidermal toxin level greatly exceeds that of the uropygial gland (Dumbacher et al. 1992), so it can be inferred that the types of lipid synthesized in the epidermis may have more affinity for the toxins. It is also possible that the epidermal cell proliferation and turnover differs from that of the uropygial gland, and that the sequestration of toxins may have a temporal relationship to the bird's toxin source (Dumbacher et al. 2004).

In conclusion, the skin of Hooded Pitohuis appears to be anatomically and histologically unremarkable, as compared with the skin of other passerines (i.e., it lacks any obvious specialization for storing or secreting toxins). We hypothesize that epidermal lipogenesis may be a pre-adaptation to sequestering lipophilic BTXs in the skin and feathers. In pitohuis, the stratum corneum and its lipids could also provide a depot for toxins that could be time-released on the basis of exfoliation of corneocytes or potential lipid hydrolysis by enzymes secreted by the cutaneous microflora. Such a depot may partially compensate for an irregular supply of dietary source of toxins, such as beetles in the genus *Choresine* (Dumbacher et al. 2004).

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