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Phylogenetic investigation of skin sloughing rates in frogs: relationships with skin characteristics and disease-driven declines

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Amphibian skin is highly variable in structure and function across anurans, and plays an important role in physiological homeostasis and immune defence. For example, skin sloughing has been shown to reduce pathogen loads on the skin, such as the lethal fungus *Batrachochytrium dendrobatidis* (*Bd*), but interspecific variation in sloughing frequency is largely unknown. Using phylogenetic linear mixed models, we assessed the relationship between skin turnover rate, skin morphology, ecological traits and overall evidence of *Bd*-driven declines. We examined skin sloughing rates in 21 frog species from three continents, as well as structural skin characteristics measured from preserved specimens. We found that sloughing rate varies significantly with phylogenetic group, but was not associated with evidence of *Bd*-driven declines, or other skin characteristics examined. This is the first comparison of sloughing rate across a wide range of amphibian species, and creates the first database of amphibian sloughing behaviour. Given the strong phylogenetic signal observed in sloughing rate, approximate sloughing rates of related species may be predicted based on phylogenetic position. While not related to available evidence of declines, understanding variation in sloughing rate may help explain differences in the severity of infection in genera with relatively slow skin turnover rates (e.g. *Atelopus*).

1. Introduction

Given the importance of amphibian skin for a multitude of physiological functions, this organ is highly diverse in its form and function [1–3]. The 'typical' amphibian exhibits highly permeable skin that leaves the organism vulnerable to desiccation in terrestrial environments and permeable to water and electrolytes in aquatic habitats [4]. However, amphibians have developed a number of physiological, structural and behavioural mechanisms to overcome these challenges [3–5]. Anurans are adapted to a wide variety of habitats and ecological niches, and morphofunctional properties of their skin have conferred tolerance to thermal and moisture extremes in many species [5]. Adaptations of amphibian skin have allowed for decreased water loss (waxy lipids [6]; cocoon formation [7]), increased water uptake (vascularized drink patch [8–10]; skin sculpturing and water channelling [11]), thermal regulation via evaporative water loss (mucous glands

[12,13]), and even potentially novel methods of thermoregulation and UV protection via coloration change (concentration of iridophores [14]). Amphibian skin is also the first barrier encountered by potential pathogens [15,16]. Thus, solely in terms of the amphibian integument, the diversity of form and function of this organ would indicate that amphibians are not uniform hosts for cutaneous pathogens.

The chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*) is a generalist pathogen that is affecting amphibians on a global scale [17,18]. It is found on over 500 amphibian species and counting; never has a single pathogen threatened such a wide diversity of species within a single class of vertebrates [19,20]. In post-metamorphic amphibians, this pathogen is restricted to the keratinized layers of amphibian skin, and despite this localization, infection can result in the disease chytridiomycosis, and mortality [21,22]. However, the degree to which host species are susceptible to infection and subsequent clinical disease is highly variable, and is probably the combined result of host and pathogen ecology and biology, community structure, and the thermal and hydric environment [18,23]. Given that this pathogen is entirely cutaneous in post-metamorphic animals, and amphibian skin is important for a range of physiological and immune defence functions, understanding the variation in skin structure and function across amphibian species is paramount.

A key aspect of amphibian skin biology is the nature of its continual renewal via routine moulting or sloughing. During this process, the outer keratinized layer of skin, or *stratum corneum*, is shed in one piece via a series of limb and body movements, after which the shed skin is routinely eaten [24]. It is assumed that this process transpires in most amphibians, and it is thought to occur anywhere from daily [25] to once a week or fortnightly [26,27]. Sloughing primarily acts in skin renewal, but may also play a role in controlling cutaneous microbial populations [27,28], and even regulating *Bd* load on the skin [29]. It has thus been suggested that sloughing rate, or the rate of epidermal turnover, could contribute to the susceptibility of amphibians to chytridiomycosis [29–31]. However, most of the research on amphibian sloughing occurred prior to the discovery of *Bd*, and only with a limited number of common laboratory species [24]. Consequently, there is a poor understanding of the variation in skin sloughing rates among amphibians, and whether this trait demonstrates phylogenetic signal.

Furthermore, the structure of amphibian skin is highly variable across species, particularly in terms of its thickness, sculpturing, number of mucosal and peptide glands, and the presence of additional structures postulated to play a role in the resistance of amphibian skin to cutaneous water loss (e.g. calcified dermal layer [3,5]). Epidermal thickness, and in particular the number of replacement layers in the epidermis, may correlate with the rate of epidermal turnover, and could indicate the level of 'moulting plasticity' within individuals or species [30]. Given amphibians infected with *Bd* demonstrate an increase in sloughing rate [31], more epidermal layers may provide the flexibility to increase sloughing rates without physiological consequence. In addition, skin thickness has been hypothesized to relate to the ecological habit of the amphibian [5], and may reflect the overall permeability of the skin to water. Understanding the variation in epidermal thickness across species could provide important insight into basic physiology and susceptibility to cutaneous pathogens.

In order to create the first database of amphibian skin structure and function, we used a recently developed non-invasive method to analyse sloughing rates in captive amphibians [31], focusing on anurans. The number of amphibian species in captivity for conservation reasons continues to grow as species at risk of extinction are collected for captive breeding programmes [32,33]. In addition to their direct purpose, these programmes are an excellent resource for better understanding the biology and ecology of these species, via remote monitoring with infrared cameras. To capture a variety of amphibian species from two areas that have experienced severe amphibian declines, this comparison focused on frog species from Australia and Central and South America [21,34,35]. Furthermore, using museum specimens, samples of ventral skin for each species were analysed for structural differences, namely epidermal thickness and the number of epidermal layers. Using a phylogenetic mixed model framework to take into account non-independent evolutionary history among species, we examined the relationship of these skin traits to characteristics of their life history, and available evidence of the susceptibility of these species to the disease chytridiomycosis.

Understanding how interspecific differences in amphibian skin structure and turnover rates change within a phylogenetic context might help us better understand the drivers of susceptibility to cutaneous pathogens. Therefore, we investigated whether sloughing rate demonstrates phylogenetic signal across anuran species, and if this rate is influenced by body size, temperature, life history or skin characteristics. Furthermore, activity time (nocturnality or diurnality) may influence anuran physiology because primarily diurnal or nocturnal frogs experience opposite ends of daily thermal variation and subsequent potential thermal and water balance stress during active periods [36,37]. Given that sloughing behaviour is also physiologically challenging [38,39], the timing of the sloughing event was compared for diurnal and nocturnal species. Finally, both skin structure and sloughing rate were investigated as predictors of overall susceptibility to chytridiomycosis among the species measured. We hypothesized that species with a thinner epidermis and slower epidermal turnover would demonstrate greater evidence of *Bd*-driven declines in wild populations.

2. Methods

(a) Measuring anuran sloughing rates

Twenty frog species were filmed at zoos and captive breeding centres to determine sloughing rates (electronic supplementary material, table S1), and sloughing rates for one species (*Rhinella marina*) were obtained from the published literature [39]. Whenever possible, anurans were filmed individually, and never more than two per enclosure. Frogs were recorded continuously with 12 600TVL weatherproof infrared cameras (model EN-CI20B-65H, Eonboom Electronics Limited) at a frame rate of 1.52 frames per second (fps). Video was recorded on either a 16-channel H.264 digital video recorder (DVR) (model MDR688ZB (AU)-E. 600TVL) or a 4-channel DVR (system model DVR-6204T), depending on the location. Monitoring amphibian sloughing via infrared video cameras has been shown to be successful previously [29,31], and sloughing behaviour is easily recognizable when viewing recordings at 16× normal speed.

Enclosures used for filming were relatively simple at all locations to aid viewing of behaviours, with a few exceptions. In general, amphibians were provided with a damp substrate of crumpled paper towels, as well as a plastic cup or half a plastic PVC pipe for shelter. Given amphibian husbandry varied depending on institutional husbandry practices, deviations from these

conditions are noted in electronic supplementary material, table S1. Amphibians were filmed for two to three weeks at each location, and temperatures were monitored with Thermochron iButtons (Maxim Integrated). With the variation in temperature requirements across species, average temperature over the entire filming period was used as a covariate in analyses.

At each location, the mass and snout–vent length (SVL), sex (if known), and unique coloration characteristics (if filmed in pairs) for each individual were recorded. At the Balsa de los Sapos, a facility dedicated to amphibian conservation at the Pontificia Universidad de Católica del Ecuador, frogs were swabbed at the start of filming to determine *Bd* infection status, given a recent case of *Bd* infection in the facility. Swabs were analysed with standard qPCR techniques [40] by Allan Pessier at the San Diego Zoo.

Videos were analysed by multiple 'viewers', and sloughing events and times were corroborated by at least one other 'viewer'. Analysed videos were used to calculate intermoult intervals (IMI, h), or the time between sloughing events, as well as slough duration (min) and timing analyses.

IMI values for *Rhinella marina* were published previously [39], and were not obtained from video footage, but rather via the traditional marking and observation method. A small amount of non-toxic waterproof zinc cream (Key-Sun Laboratories Pty Ltd, NSW, Australia) was applied to the dorsal surface and animals were checked twice-daily to record the disappearance of a mark (indicating sloughing had occurred). Marks were reapplied once the disappearance of a mark occurred. This method works well for terrestrial toads that have fairly dry skin [27,39], but does not provide information on the duration and exact timing of the sloughing behaviour, which was therefore not available for this species.

(b) Epidermal structure

Ventral skin samples were collected from preserved specimens (fixed in 10% neutral buffered formalin, stored in 70% ethanol) at museums and captive breeding centres. All skin samples were taken from the ventral pelvic area, as this area is most often infected with *Bd* [41]. Samples were taken from one to five individual specimens per species. Samples were processed, embedded, sectioned at 5 µm thickness, and stained with haematoxylin and eosin. Samples that were preserved poorly, or demonstrated evidence of skin abnormality (disease, etc.) were excluded (see electronic supplementary material, appendix S1 for the list of specimens used in analyses). Images were analysed in ImageJ (version 1.48 [42]) to determine epidermal thickness (micrometres) and the average number of epidermal cell layers per species.

(c) Phylogenetic relationships

A phylogenetic tree of all species analysed was obtained from the Open Tree of Life [43], accessed via the R package 'rotl' [44]. Closest relatives were used for sub-species or species complexes not found within the tree (*Atelopus* sp. [spumarius-pulcher complex] and *Litoria verreauxii alpina*), and Grafen's arbitrary branch lengths were used for tree creation [45].

(d) Susceptibility measures

To categorize species based on their known susceptibility to chytridiomycosis, two criteria were used. First, species were classified based on the evidence for chytridiomycosis-related declines, as cited in the IUCN Red List [46], published papers, grey literature and personal communications (this classification scheme was modelled after [47]): (1) direct evidence of *Bd*-driven declines in the published literature, (2) chytridiomycosis listed as a threat in the IUCN Red List or (3) no evidence of *Bd*-driven declines (see electronic supplementary material, appendix S3). In addition, for a subset of species in which published experimental *Bd* infection studies existed ($n = 12$), mortality rates were used as a susceptibility measure (see electronic supplementary material, appendix S4). However, it is important to acknowledge that mortality rates from exposure studies may not be indicative of susceptibility to declines in nature. In addition, exposure studies used a wide range of inoculum doses and *Bd* isolates, and thus provides only a rough indication of potential susceptibility to this pathogen (see electronic supplementary material, appendix S4 for details of dose and isolate information).

(e) Species life-history classification

Given skin characteristics, such as skin resistance to evaporative water loss, have been associated with life history in amphibians [48], species were classified by ecological habit (B, burrowing; A, arboreal; T, terrestrial; SA, aquatic/semi-aquatic), ecological group (S, stream associated; P, pond breeding; E, ephemeral water breeder; T, terrestrial breeder or a combination of two, e.g. E/T or E/P), and prevailing activity time (either nocturnal or diurnal). These classifications were based on those used by Murray *et al.* [49] and species were categorized based on information from online databases (AmphibiaWeb [50] and the IUCN Red List [46]), as well as personal communication with experts.

(f) Statistical analyses

All statistical analyses were performed in R [51]. Phylogenetic linear mixed models (PLMMs) were implemented using restricted maximum-likelihood estimation (REML) in ASReml-R [52], which can account for multiple measurements on the same individuals over time, and phylogenetic non-independence between species (function 'asreml', package 'asreml'). All models included individual *Frog ID* nested within *Species* and a phylogenetic variance-covariance matrix constructed from the phylogenetic tree, as random effects. A Wald-type *F*-test was used to test for the significance of fixed effects [53], and the significance of random effects was determined using likelihood ratio tests [54,55]. Phylogenetic heritability, which is equivalent to the more widely used lambda (λ) [56], was used as an estimate of phylogenetic signal, and was calculated as the proportion of the variance in the trait, conditioned on the fixed effects [57], which is explained by the relationship among taxa as given by the phylogeny [58]. Approximate standard errors for the estimate of phylogenetic heritability were calculated using the R pin function [59].

Given that temperature affects sloughing rates [28], we first tested the effect of the average, minimum and maximum temperatures experienced by the amphibians during the filming periods on sloughing rate. Next, the relationship between sloughing rate and (a) slough duration and SVL, (b) ecological group, ecological habit and activity time (the time of day at which each species is active, e.g. diurnal or nocturnal), (c) skin thickness and (d) the number of epidermal layers was tested. Finally, we assessed the relationship between average sloughing rate (by species) and the evidence for *Bd*-driven declines or per cent mortality from published studies, taking into account ecological group. We also assessed the relationship between percentage mortality and the number of epidermal layers, taking into account life stage at exposure. All continuous variables were log or natural-log transformed to meet the requirement of normality, and models were reduced using likelihood ratio tests [54,55].

3. Results

(a) Sloughing behaviour and intermoult interval

Overall, sloughing rates were measured for 21 anuran species from eight different families, originating from Asia (1), Central/South America (11) and Australia (9) (see electronic supplementary material, appendix S2). On average, 59.7

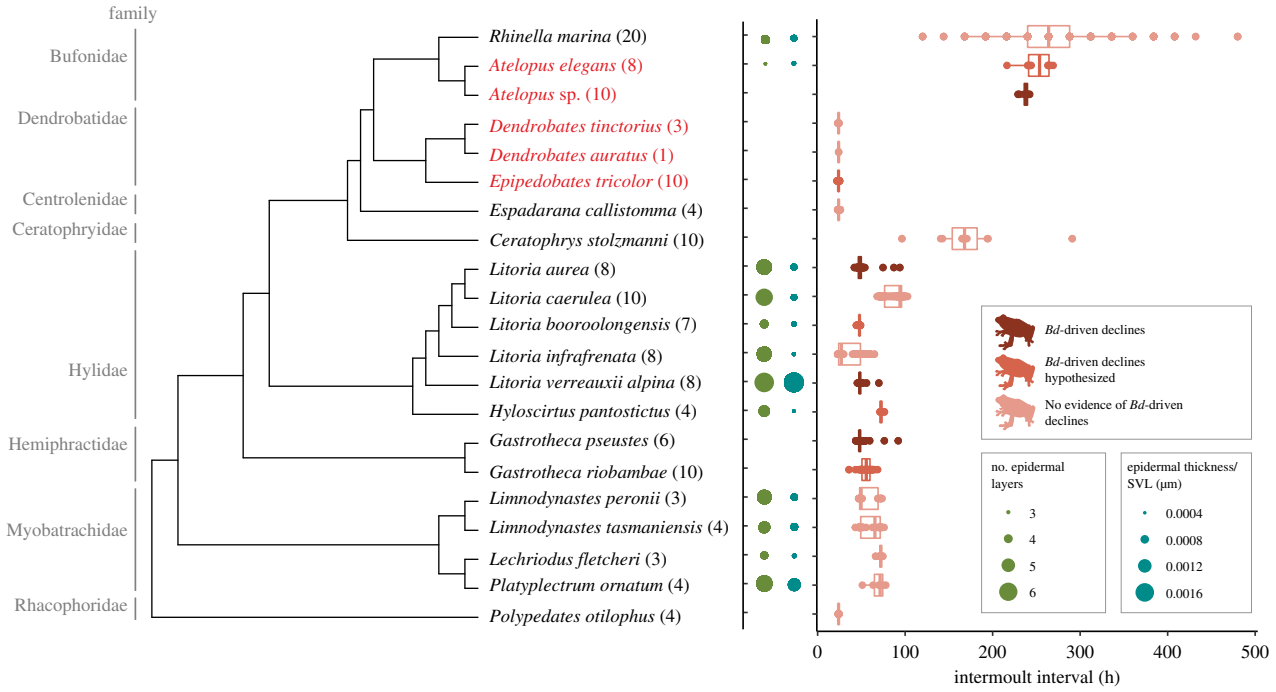

Figure 1. Phylogenetic relationships between 21 frog species for which IMI, or the time between sloughing events, were measured (represented by boxplots on the right, sample size in parentheses next to each species name). Boxplot colours indicate the evidence for *Batrachochytrium dendrobatidis* (*Bd*)-driven declines, categorized by direct evidence of *Bd*-driven declines, *Bd*-driven declines indicated by the IUCN Red List, and no evidence of *Bd*-driven declines (shades of red). Green circles represent the average number of epidermal layers in museum specimen for those species, while teal circles represent average epidermal thickness divided by snout–vent length of the specimen (micrometre). Species names in orange indicate diurnal species, while the remainder are nocturnal species. Branch lengths are Grafen's arbitrary branch lengths.

sloughing events were recorded per species, with a minimum of 3 and a maximum of 359. In general, species that sloughed more often allowed for the recording of more sloughing events. IMI ranged from 22 h (daily) to 480 h (every 20 days), with a mean interval of 119.5 h (median: 71.7 h) across all species (figure 1).

Sloughing behaviour, or the physical removal of the *stratum corneum* during the sloughing process, was fairly similar across the anurans studied, although the duration and timing of the behaviour differed substantially. Generally, sloughing behaviour followed previous reports [24,31], in which a series of fore and hind limb movements, side contractions and opening and closing of the mouth, aided the movement of the *stratum corneum* into the mouth. Most animals inflated with air before the sloughing process began, presumably to facilitate the splitting of the *stratum corneum* on the dorsum. Of note, burrowing species demonstrated slightly different sloughing behaviour, which involved less physical pushing of the skin with fore and hind limbs, given the comparably short length of their legs, and more lateral movement of the entire body back and forth. In addition, some species consistently sloughed from an elevated position, usually from a wall of the enclosure (e.g. *Atelopus* spp.). Furthermore, the timing of sloughing was strongly related to the activity pattern of that species as observed on the videos, with primarily nocturnal species typically sloughing in the early evening or at night, and primarily diurnal species sloughing in the early morning ($F_{1,8.1} = 29.45$, $p = 0.0006$; electronic supplementary material, table S2; figure 2).

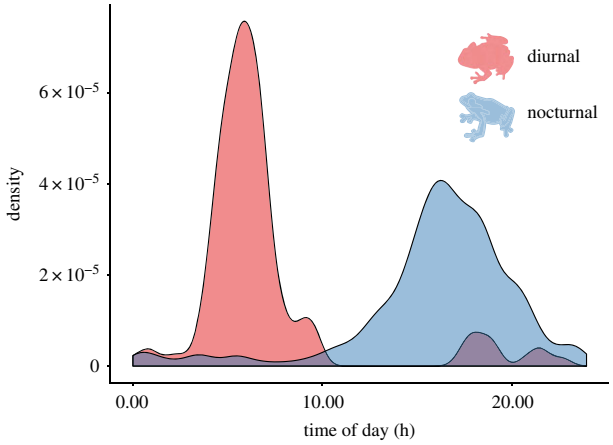

Figure 2. Time of day sloughing events occurred for diurnal ($n = 5$) and nocturnal ($n = 15$) anuran species, expressed as kernel density plots. (Online version in colour.)

(b) Bd status of frogs

Two of the nine species filmed at the Balsa de los Sapos tested positive for *Bd*. *Bd* prevalence was 20% in *Gastrotheca pseustes* ($n = 2/6$) and 30% in *Ceratophrys stolzmanni* ($n = 3/10$). Sloughing rate was consistent across infected and uninfected individuals, and infection load was very low in both species (*G. pseustes*: 7.2 ± 13.4 s.d.; *C. stolzmanni*:16.0 ± 3.5 s.d. zoospore equivalents [ZE]). Previous work indicates that sloughing rates do not increase in *Bd*-infected animals until high infection loads are reached [31], and these animals were not demonstrating clinical signs or variation in behaviour. Captive frogs at Taronga Zoo and the San Diego Zoo are tested on a regular basis, and no records of chytridiomycosis had occurred in either facility before, during or after the filming periods. All frogs filmed at The University of Queensland were regularly tested for *Bd* infection, and all remained *Bd* negative during filming.

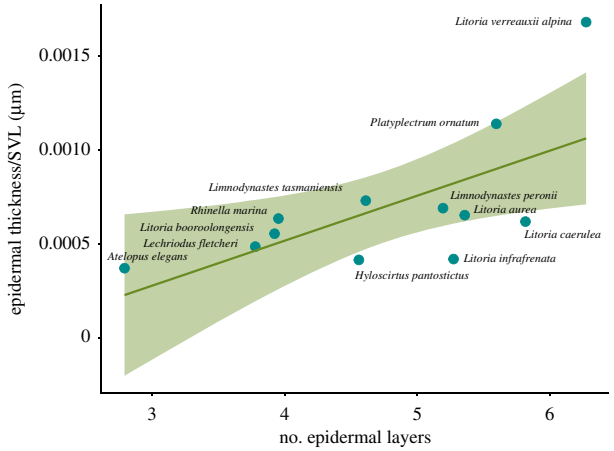

Figure 3. A positive association between epidermal thickness/SVL (micrometres) and the number of epidermal layers in 12 anuran species, measured from museum specimens. Shaded area indicates standard error. (Online version in colour.)

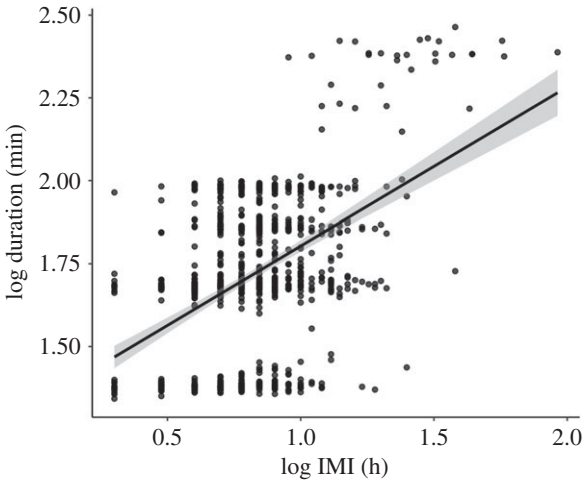

Figure 4. Relationship between slough duration and IMI across 20 amphibian species. Shaded area indicates standard error.

(c) Skin structure

After removing skin samples that were poorly preserved or demonstrated evidence of disease, skin structure was analysed based on multiple sections from one to four specimens per species (mean: 2.6). Ventral epidermal thickness across amphibian species ranged between 9.4 and 75.3 µm (mean: 31.4 ± 14.4 s.d.). Epidermal thickness, standardized by SVL of the specimen (micrometre), was positively correlated with the number of epidermal layers ($F_{1,9.5} = 9.4$, $p = 0.013$, figure 3), which ranged between 2 and 7 layers (mean: 4.8 ± 1.0 s.d.). The structure of the ventral skin varied across species, but generally followed the structure of the typical anuran epidermis, containing one to two layers of *stratum corneum*, one to four layers of *stratum granulosum*, and one to two layers of *stratum basal*. The dermal layer varied greatly in thickness across species, and contained mucosal and peptide glands within the *stratum spongiosum*, followed by the *stratum compactum*. The greatest variation existed in the number of mucous and peptide glands, the amount of chromatophores in the *stratum spongiosum*, and the sculpturing of the ventral skin (electronic supplementary material, figures S1 and S2).

(d) Relationship between intermoult interval and other life-history and skin structural traits

Overall, IMI across all species was not influenced by mean, minimum or maximum temperature during the measurement periods of each species (mean: $F_{1,17.1} = 1.78$, $p = 0.91$, min: $F_{1,16.7} = 0.56$, $p = 0.88$, max: $F_{1,17.1} = 1.37$, $p = 0.26$; electronic supplementary material, table S3). Furthermore, there was no relationship between IMI and SVL ($F_{1,151.5} = 0.001$, $p = 0.97$, electronic supplementary material, table S3), epidermal thickness/SVL ($F_{1,9.8} = 0.02$, $p = 0.89$, electronic supplementary material, table S3), or the number of epidermal layers ($F_{1,8.1} = 0.026$, $p = 0.88$, electronic supplementary material, table S3). However, IMI did demonstrate strong phylogenetic signal in all of these models (range: $\lambda = 0.91–0.97$, s.e. = 0.008–0.11), with species in the family Bufonidae demonstrating the longest IMIs between 7 and 20 days, and species within Dendrobatidae almost exclusively sloughing every 24 h, demonstrating some of the shortest IMIs (figure 1). IMI was positively associated with sloughing duration when average temperature was taken into account ($F_{1,1244.7} = 11.08$, $p = 0.001$; figure 4; electronic supplementary material, table S4), indicating that frogs that slough less often take longer to complete the sloughing process. Across all species, the duration of the sloughing event varied between 2 and 92 min (mean: 8.2 ± 7.5 s.d.). IMI was also significantly different across ecological group ($F_{4,12} = 9.14$, $p = 0.0013$; electronic supplementary material, figure S3), between nocturnal and diurnal species within the stream-associated breeders ($F_{1,12} = 6.74$, $p = 0.023$; electronic supplementary material, figure S3), and across ecological habit ($F_{3,12} = 3.58$, $p = 0.047$; electronic supplementary material, table S5). In this model, phylogenetic signal of IMI is low ($\lambda = 1.4 \times 10^{-6}$, s.e. $= 4.9 \times 10^{-7}$; electronic supplementary material, table S5). Estimates of λ are conditioned on the fixed effects [57,60], so it is likely that once the fixed effects of ecological group and habit are accounted for, there is no phylogenetic signal remaining.

(e) Intermoult interval, skin characteristics and susceptibility

Evidence for *Bd*-related declines in the wild was not significantly associated with average IMI by species, but was significantly associated with the ecological group ($F_{4,8.8} = 4.09$, $p = 0.036$; electronic supplementary material, table S6), with stream- and pond-associated breeders demonstrating greater evidence of *Bd*-driven declines. In addition, percentage mortality was not associated with IMI when ecological group and life stage at exposure were taken into account ($F_{1,8.2} = 0.004$, $p = 0.21$; electronic supplementary material, table S7), but this only included a subset of species ($n = 12$) for which published *Bd* exposure studies exist. Percentage mortality from *Bd* exposure for this group of species was associated with the number of epidermal layers ($F_{1,10.1} = 5.14$, $p = 0.045$; electronic supplementary material, table S8), with higher mortality rates associated with more epidermal layers, but this was based on a small sample size ($n = 10$) for which we had both published exposure studies and skin measurements. Both life stage ($F_{1,13.6} = 15.15$, $p = 0.0017$; electronic supplementary material, table S8) and ecological group ($F_{3,12.6} = 2.55$, $p = 0.026$; electronic supplementary material, table S8) were also significant in this model, with juvenile frogs and ephemeral water body breeders demonstrating higher mortality rates overall.

4. Discussion

Amphibian skin is a highly diverse organ, and adaptations of generally permeable amphibian skin have contributed to the extraordinary range of habitats and ecological niches they fulfil [3]. Understanding the variation in basic skin structure and function across a variety of species within a phylogenetic context can provide insight into the effects of these differences on susceptibility to a generalist cutaneous pathogen such as *Bd*. Across 21 amphibian species from eight different families, we found substantial variation in the rate of epidermal turnover, with some species sloughing every day, and other species demonstrating larger individual variation and sloughing as slowly as every other week. This variation demonstrated a strong phylogenetic signal, indicating that a species's sloughing rate tended to be more similar to closely related species than to distantly related species [61]. Overall, IMI was not influenced by body size, temperature or skin characteristics such as epidermal thickness or the number of epidermal layers. Interestingly, IMI was different across ecological groups and habits, and between nocturnal and diurnal species of the stream-associated breeders, but the number of species in each of these groups for this study may prevent further interpretation. Finally, the evidence for disease-related declines across all species was not associated with IMI, but again demonstrated significant differences across ecological groups, with stream and pond-associated breeders demonstrating greater evidence of *Bd*-related declines. Within the species examined, there was no clear association between sloughing rate and susceptibility to chytridiomycosis. However, an understanding of epidermal turnover in amphibian genera particularly affected by *Bd*, such as *Atelopus*, may help focus conservation efforts.

Within the range of amphibians studied, concentrated in Australia and Central/South America (and one species from Asia), we found IMI demonstrated very strong phylogenetic signal. This may indicate that the control and regulation of sloughing behaviour are strongly phylogenetically conserved, despite differences in habitat and ecological group among related species. While the animals used in this study have varied rearing histories, given the small intraspecific variation in sloughing rate, and the high phylogenetic signal, it would appear that this trait is not evolutionarily labile. Species in the Dendrobatidae and Centrolenidae families exhibited fast sloughing rates, with sloughing occurring every day. Conversely, species in the Bufonidae family demonstrated the longest IMIs. This family demonstrates varying degrees of dependence on environmental moisture, and wide variation in thermal tolerances, which correlate with distributional range size [62]. For example, bufonids in the genus *Atelopus* are often range-restricted and found solely in regions with high humidity, while the invasive *Rhinella marina* has demonstrated tremendous capacity to survive in a wide variety of habitats worldwide [62]. Of note, species in the genus *Atelopus* have been particularly devastated by the disease chytridiomycosis, with disease-related declines occurring across the genus in Central and South America [63]. If sloughing rate demonstrates strong phylogenetic signal, it is likely that other *Atelopus* species demonstrate relatively slow epidermal turnover as well. This has implications for understanding the progression of disease in these species, as well as the regulation of cutaneous symbiotic microbiota.

Previous work has demonstrated that sloughing can regulate not only *Bd* load [29], but also cultivable cutaneous microbial communities [27,28]. Given the potential importance of symbiotic cutaneous bacteria in innate immune defence against *Bd* infection [64], understanding the sloughing rates of imperilled amphibian species may aid conservation actions. For example, *Atelopus zeteki*, like many *Atelopus* species, has experienced widespread declines in Central America due to epidemics of chytridiomycosis, and consequently has been bred in captive assurance colonies to avoid extinction in the wild [33,63]. The realization that this species may have relatively slow skin turnover sheds light on why they may be considered *Bd* 'supershedders' [65], reaching high infection intensities and developing clinical chytridiomycosis quickly. While previous attempts to inoculate *A. zeteki* with beneficial bacteria active against *Bd* were ineffective [66], knowledge of sloughing rates could help increase chances of success. Understanding that these species probably slough infrequently may help pinpoint when to best bioaugment their skin with beneficial anti-*Bd* bacteria [66,67], as addition of the bacteria immediately after sloughing may allow for colonization when resident bacterial and potential pathogen populations are low [28].

Interestingly, the activity time of each species was associated with the time of the sloughing event, with nocturnal species sloughing in the evening or at night, and diurnal species sloughing in the early morning. Sloughing usually occurred before the daily activity period began in all species, which may be adaptive in that frogs would usually slough in day or night refuges (depending on whether they are nocturnal or diurnal), and then commence the active period. Frogs typically select refuges that reduce the rates of cutaneous water loss [68,69], and frogs demonstrate high rates of evaporative water loss during sloughing [70]. Thus, sloughing within a refuge could allow frogs to potentially avoid thermal or hydric extremes during the sloughing period, or reduce the risk of predation. Conversely, the timing of sloughing may be related to the sleep patterns of diurnal and nocturnal species, given that sloughing is an active process that results in a series of hormonal changes within the individual.

IMI was not associated with temperature, amphibian body size (SVL), epidermal thickness or the number of epidermal layers. Although the sloughing rate is positively correlated with temperature within a species [27,28], the range of mean temperatures experienced by each species in this study was fairly narrow (18.8–25°C), which helped fortify the comparison across species. Thus, the trends observed across species were probably not an artefact of differences in temperature during the measurement period. Furthermore, sloughing rate was not explained by amphibian body size or skin structure. Interestingly, IMI was positively associated with the duration of the sloughing event, when taking into account mean temperature. Frogs with high rates of skin turnover may have evolved a coincidently fast sloughing behaviour to compensate for the frequency of this physiologically vulnerable period. During sloughing, amphibian skin increases in permeability to both water and electrolytes [38,39,70], and the risk of predation is likely greater, thus high rates of sloughing may be physiologically stressful. For example, green tree frogs (*Litoria caerulea*) have been shown to have the highest rates of cutaneous water loss during the sloughing period [70]. Conversely, sloughing rate and duration may be inherently linked traits, but the genetic mechanisms governing sloughing are unknown.

Sloughing rate was significantly different across the ecological group, habit and activity time, with ephemeral/pond breeders demonstrating slower epidermal turnover than terrestrial/ephemeral breeders, and diurnal stream breeders

demonstrating higher sloughing rates than nocturnal stream-associated breeders. This categorization is interesting, but may be more related to the number of species in each of these categories in this study, and the number of species in each ecological group per family. Also, it may be that there is a high phylogenetic signal in ecological group and habit as well, as has been demonstrated in other macroecological variables in amphibians [71]. Thus, a larger sample size across a greater range of amphibian species may be needed to tease apart variation in IMI across ecological group and habit.

We did not find an association between evidence of *Bd*-driven declines and sloughing rate or skin structure, although we did find greater evidence of *Bd*-driven declines in pond and stream-associated breeders. Ecological group and reliance on water have demonstrated a strong correlation with *Bd*-related declines in the previous work [49,72], so this finding is not surprising. However, the classification of amphibians in this study based on *Bd*-driven declines is geographically biased by the available information for each species, with more publications and *Bd* exposure studies for Australian species compared with species from South America and Asia. Frogs with a greater number of epidermal layers demonstrated higher mortality rates after *Bd* exposure, but this was only in a subset of species for which these traits could be examined. Furthermore, juvenile amphibians demonstrate higher mortality rates when exposed to *Bd* than subadults/adults, and not all species had representative exposure studies for both life stages. Thus, additional information regarding susceptibility to chytridiomycosis in each species studied will help further elucidate the role of sloughing rate or skin structure in that susceptibility. Regardless, given the high level of phylogenetic signal in sloughing rate, this work provides the first framework for predicting the sloughing rates of related species, which previously was a little-known aspect of amphibian skin physiology and behaviour. In doing so, we may be able to better customize models of *Bd* growth on amphibian skin, taking into account how often resident and potentially pathogenic organisms are removed from the skin via sloughing for a particular species. For example, recent work indicates that individual-based models [73] or integral projection models [74] can be useful for modelling infection dynamics in pathogens such as *Bd* that demonstrate both micro- and macroparasite characteristics. Sloughing rates can be incorporated into such models to better predict how *Bd* will grow on individual hosts, leading to more accurate predictions of host survival and population persistence in the field.

The understanding of basic amphibian biology and physiology within a phylogenetic context can inform conservation efforts. We demonstrate there is a strong phylogenetic signal in amphibian skin sloughing rates, and this can improve our understanding of cutaneous disease progression in focal species for conservation mitigation strategies.

Ethics. All methods involving animals were approved by and carried out in accordance with the guidelines and regulations of permit SBS/452/12/URG issued by the University of Queensland Animal Welfare Committee (AWC) and permit WISP12218412 issued by the Queensland Environmental Protection Agency. All external institutions agreed to participate in the study to allow for the filming of frogs under the University of Queensland AWC. In addition, filming at Taronga Zoo was also approved by the Animal Ethics Committee of the Taronga Conservation Society Australia, protocol 4d/10/13.

Data accessibility. All data analysed in this study are available in the electronic supplementary material.

Authors' contributions. M.E.B.O., R.L.C. and C.E.F. conceived and designed the study; M.E.B.O., P.S.H., M.S.M., A.M.-V., A.P.P., N.C.W. and P.J.B. carried out experimental work; M.E.B.O. and C.R.W. performed data analysis; M.E.B.O. wrote the manuscript, and all authors contributed to revising the manuscript.

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