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TITLE: Percutaneous absorption between frog species: variability in skin may influence delivery of therapeutics

RUNNING TITLE: Interspecific variability in absorption through frog skin

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ABSTRACT

Frogs have permeable skin, so transdermal delivery provides a practical alternative to traditional dosing routes. However, little is known about how frog skin permeability differs interspecifically, and there are different reported clinical outcomes following topical application of the same chemical in different frog species. This study collated in vitro absorption kinetic data previously reported for two frog species: the green tree frog (*Litoria caerulea*) and the cane toad (*Rhinella marina*), and used linear mixed-effects modelling to produce a model of absorption. Histology of skin samples from each species was performed to observe morphological differences that may affect absorption. Absorption kinetics differed significantly between species, with the LogP of the applied chemical a

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27 better predictor of permeability than molecular weight. Application site also influenced
28 permeability, with dorsal permeability consistently higher in cane toads. Ventral permeability was
29 more consistent between species. Skin thickness differed between species and skin regions, and this
30 may explain the differences in absorption kinetics. Guidelines for selecting chemicals and dosing site
31 when treating frogs are presented. The permeability differences identified may explain the poor
32 reproducibility reported in the treatment of disease across frog species, and reinforces the
33 importance of considering inter-species differences when designing therapeutic treatments for
34 frogs.

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36 KEYWORDS: Animals; Skin absorption; Anura; Permeability; Administration, cutaneous

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38 Compared to mammals, birds and reptiles, frog skin is highly permeable, providing an ideal route for
39 easy, low-stress administration of therapeutics. This is attributed to frogs' very thin stratum
40 corneum, which often consists of only 1–2 cell layers. Frog skin also shows regional variation in
41 thickness and vascularity, both within individual frogs and between species (P. J. Bentley & Yorio,
42 1976; Toledo & Jared, 1993); these differences are expected to influence the relative absorption of
43 chemicals. Skin thickness appears to be related to a frog's primary habitat, with arboreal frogs
44 typically having a very thick dorsal skin to prevent desiccation, whereas terrestrial and aquatic
45 frogs—which are equally exposed to moisture from all sides—often have a more consistent skin
46 covering across the entire body (P.J. Bentley & Main, 1972; P. J. Bentley & Yorio, 1976; Christensen,
47 1974; Yorio & Bentley, 1977).

48 Chytridiomycosis, a deadly fungal infection of the keratinised epidermis, has caused mass mortality
49 and extinction in frog species worldwide (Scheele et al., 2019). While many topical antifungal have
50 been investigated for management of chytridiomycosis, only miconazole (Nichols, Lamirande,
51 Pessier, & Longcore, 2000), itraconazole (Brannelly, Richards-Zawacki, & Pessier, 2012; Jones et al.,
52 2012; Nichols et al., 2000; Tamukai, Une, Tominaga, Suzuki, & Goka, 2011), chloramphenicol (Young,
53 Speare, Berger, & Skerratt, 2012), terbinafine (Bowerman, Rombough, & Weinstock, 2010), and
54 voriconazole (Martel et al., 2011) have been reported to provide successful treatment. However, a
55 consistently-effective treatment remains elusive, as repeat experiments in different frog species
56 often result in different therapeutic outcomes (Berger, Speare, Pessier, Voyles, & Skerratt, 2010;
57 Roberts et al., 2018). As the therapeutic outcome differs even when controlling for both chemical
58 and formulation, it is likely that differences in percutaneous absorption due to inter-species
59 variability in skin structure is the reason for these inconsistent results.

The current study investigated how differences in skin thickness in arboreal and terrestrial frogs influence percutaneous absorption. Previously-collected in vitro percutaneous absorption data for three model chemicals—caffeine, benzoic acid, and ibuprofen—in the arboreal frog *Litoria caerulea* (Llewelyn, Berger, & Glass, 2018) and the terrestrial toad *Rhinella marina* (Llewelyn, Berger, & Glass, 2019) were combined and reanalysed using R (R Core Team, 2016) to create a single model of absorption. To determine the relationship between absorption (permeability co-efficient; K_p) and species, skin region of application, animal weight, sex, and physicochemical properties of the applied chemical (logP or molecular weight), separate linear mixed-effects models with different combinations of predictor variables and interactions between predictor variables were created. Linear mixed-effects models were fitted by maximum likelihood using the lme function in the nlme package (Pinheiro, Bates, DebRoy, & Team, 2017). As data included three chemicals, molecular weight (MW) and logP could not both be included as covariates in the same model, and so were investigated individually. All models included the individual animal as a random effect, and models that allowed and did not allow for heterogeneity in data were examined. Models were compared using ANOVA, Akaike's Information Criterion, and residual plots were examined to determine best fit. Final model selection was based on a combination of the above goodness-of-fit analyses and parsimony, and the model was re-fitted using restricted maximum likelihood to produce the final absorption model.

Fresh skin samples for histology were obtained from adult *R. marina* and *L. caerulea* used during the in vitro absorption studies (Llewelyn et al., 2018, 2019). All samples were collected in accordance with Animal Ethics A2222 from James Cook University, Australia. Briefly: full-thickness dorsal and ventral skin samples were excised from five animals of each species immediately following euthanasia in a bath of 0.2% w/v ethyl 3-aminobenzoate methanesulfonate solution (MS-222; Aldrich Chemistry) buffered to pH 7.3 with sodium bicarbonate. Samples for histological analysis were immediately rinsed in Amphibian Ringer's solution (113 mM sodium chloride, 2 mM potassium chloride, 1.35 mM calcium chloride, and 2.4 mM sodium bicarbonate), and preserved in 4% phosphate-buffered formaldehyde. The skin was then dehydrated and processed for histology using standard methods to produce 5 μ m sections on slides stained with haematoxylin and eosin.

Two top models, one using MW and the other using logP, were identified for prediction of K_p . Regardless of physicochemical parameter used, both top models found that species and region of application significantly influenced K_p . While statistical comparison of the top models showed no significant differences, examination of the residual plots showed that the model using logP fit the

data better than the MW-containing model. Thus, the final model for absorption includes logP (Table 1). Analysis of the final model showed that species and region of application significantly influenced K_p , both alone and through interactions with each other. Ventral absorption was more consistent between species, whereas dorsal absorption was significantly different (higher in *R. marina*) for all chemicals investigated.

The histology collected in this study supports previous descriptions of skin structure in *L. caerulea* and *R. marina* (Ohmer et al., 2019; Schwinger, Zanger, & Greven, 2001; Willens et al., 2006).

Representative dorsal and ventral skin sections from *L. caerulea* and *R. marina* are presented in Figures 1 and 2, respectively. A key difference between species is relative skin thickness, and this is likely to influence absorption. *L. caerulea* has a much thinner ventral dermis than *R. marina*, however the ventral epidermis is slightly thicker, with a similar trend observed for the dorsal skin. These findings concur with those of Ohmer et al. (2019), in which the ventral pelvic epidermis was reported to be thicker in *L. caerulea* (average 6 cell layers thick) than in *R. marina* (average 4 cell layers thick). In terms of glandular distribution, granular glands were present dorsally in both species, however mucus glands were only present in *L. caerulea*. Granular glands appear less common in the ventral skin of *L. caerulea*, and were not observed in *R. marina*. Finally, both species have prominent epidermal sculpturing (verrucae) of the ventral skin, with this sculpturing extending to the dorsal skin in *R. marina* only. Such sculpturing has previously been described in terrestrial and arboreal species (Christensen, 1974; Lillywhite & Licht, 1974; Toledo & Jared, 1993), and is thought to facilitate water uptake and distribution by providing an increased surface area for absorption and channelling water to other skin surfaces.

The finding that absorption was significantly higher dorsally in *R. marina* than in *L. caerulea* for all chemicals can be explained by considering the histology of the dorsal skin in both species. Although *L. caerulea* full-thickness skin is slightly thinner than *R. marina*, the lipophilic epidermis is thicker. Further, the mucus glands in the dorsal epidermis of *L. caerulea* provide the skin surface with a lipid coating (Keith Christian & Parry, 1997; K. Christian, Parry, & Green, 1988), which provides an additional diffusional layer. The more consistent ventral absorption between the species can also be explained by the skin structure. Both species have a similarly-thick ventral epidermis, and verrucae which increase the surface area of skin available for chemical absorption. These findings can be used to make some general recommendations when considering therapeutic chemicals for application to the skin of frogs (Table 2).

Returning to the conundrum of inconsistent outcomes following application of chemicals to the skin of frogs for treatment of chytridiomycosis: these findings explain why such inconsistencies have occurred. Most of the antifungal agents reported to be efficacious in treating chytridiomycosis are

highly lipophilic – with logP values >5.6. Using the model produced in this study to estimate the permeability of agents in this logP range, a minimum 7-fold difference in K_p between species is predicted when such chemicals are administered to dorsal skin. In a bath formulation, as is commonly used for frogs, both the dorsal and ventral skin surfaces are exposed to chemical. If equal exposure occurs on both surfaces, this equates to at least a 3.5-fold difference in the absorption rate between the two species. This magnitude of difference in absorption would significantly impact on serum concentrations in vivo, and may well explain the inconsistent results of clinical trials for chytridiomycosis. In vivo studies in frogs that investigate regional differences in absorption of chemicals with a range of LogP values will confirm these findings. Importantly, the chemicals used to create the model produced herein only cover a LogP range of – 0.07 through 3.97. However, most therapeutic substances typically used in frogs are likely to be within this range. Studies that investigate the absorption of more highly-lipophilic and hydrophilic chemicals will permit extension of the model.

This study emphasizes the importance of considering inter-species differences, especially in terms of relative skin thickness, when selecting chemicals and dosing regimens in frogs. The model presented provides the first attempt to mathematically relate variability in skin structure between frog species to percutaneous absorption, and may be used to guide the selection of therapeutic candidates for use in frogs.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflict of interests to disclose.

AUTHOR CONTRIBUTION STATEMENT

TL, LB and BG contributed to study conception and design, manuscript drafting and revision, and approval of final manuscript. Data collection and analysis were performed by TL.

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TABLES

Table 1: Equations to predict permeability coefficient (K_p) through either dorsal or ventral skin, in cane toads (*Rhinella marina*) or green tree frogs (*Litoria caerulea*)

Species	Skin Region	Equation	Standard deviations	
			Intercept	Residual
<i>L. caerulea</i>	Dorsal	$K_p = 0.000930 - 0.000081\text{Log}P$	0.000633	0.000907
<i>L. caerulea</i>	Ventral	$K_p = 0.005178 - 0.000356\text{Log}P$		
<i>R. marina</i>	Dorsal	$K_p = 0.003805 - 0.000081\text{Log}P$		
<i>R. marina</i>	Ventral	$K_p = 0.003967 - 0.000356\text{Log}P$		

Table 2: Considerations when selecting chemicals and dosing routes for therapeutic use in arboreal and terrestrial frogs

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- 1 When selecting a chemical (therapeutic agent) for use in multiple species, consider the impact of interspecies variability in absorption, and the interplay of application site and relative lipophilicity of the chemical on absorption.
 - 2 If using dorsal application, selecting drugs with a $\log P \sim 2$ will minimise interspecies variability in absorption.
 - 3 Ventral application of chemicals generally gives higher, more consistent absorption between arboreal and terrestrial frog species. However, restricting application to only the ventral surface is difficult. Additionally, ventral application results in higher absorption, which may not be ideal if overdose and/or toxicity is a concern.
 - 4 If in doubt, selection of a drug with $\log P \sim 2$ will minimise the difference in absorption between dorsal and ventral surfaces.
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FIGURE LEGENDS

Figure 1: Histological sections of *Litoria caerulea* skin. a) dorsal skin, 5x magnification; b) ventral pelvic skin, 5x magnification; c) dorsal skin 40x magnification; d) ventral pelvic skin 5x magnification

Figure 2: Histological sections of *Rhinella marina* skin. a) dorsal skin, 5x magnification; b) ventral pelvic skin, 5x magnification; c) dorsal skin 40x magnification; d) ventral pelvic skin 5x magnification

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