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Effects of Skin Region and Relative Lipophilicity on Percutaneous Absorption in the Toad *Rhinella marina*[1]

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Abstract

Owing to the dynamic interaction between frog skin and the environment, xenobiotics in frog habitats are of particular concern, and knowledge of percutaneous absorption in frog skin is necessary for risk-mitigation purposes. Baseline transdermal kinetics in adult aquatic and arboreal frog species have recently been reported; however, there is little information regarding absorption kinetics in adult terrestrial species. The present study investigated the in vitro absorption kinetics of 3 model chemicals—caffeine, benzoic acid, and ibuprofen—through different skin regions in the terrestrial toad *Rhinella marina*. Caffeine flux was consistently higher than that of the other 2 chemicals ($p < 0.001$), whereas the fluxes of the moderately and highly lipophilic chemicals (benzoic acid and ibuprofen) were similar, regardless of skin region. When considering individual chemicals, caffeine demonstrated increased flux through the ventral pelvic skin compared with the ventral thoracic or dorsal skin regions. Flux did not differ between skin regions for either benzoic acid or ibuprofen. These findings have implications for management of environmental contamination in frog habitats because many environmental xenobiotics are of moderate to high lipophilicity and would be expected to be equally absorbed from all skin surfaces in terrestrial toads.

Keywords: Absorption, Amphibians, Octanol–water partition coefficient, Toxicokinetics, Wildlife toxicology, Skin

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INTRODUCTION

Frog skin is highly permeable, and this property may be detrimental when a frog is exposed to environmental xenobiotics or exploited for therapeutic purposes. However, little is

known about the pharmacokinetics of percutaneous absorption through frog skin, leading to the recent recommendation by the European Food Safety Authority (EFSA Panel on Plant Protection Products and Their Residues et al. 2018) that 100% dermal absorption of substances should be expected in amphibians, in the absence of any scientific data. Although it is well established that the physicochemical properties of a chemical will influence its rate and extent of absorption through mammalian skin, there is scant evidence as to how these properties influence transdermal pharmacokinetics through frog skin. Llewelyn et al. (2016) reviewed the physicochemical and skin properties that are likely to influence transdermal pharmacokinetics through frog skin, based on known frog skin physiology. Reviewing the reported outcomes for transdermal treatments and in vitro pharmacokinetic experiments in frog skin, they concluded that the relative lipophilicity of a chemical being applied to the skin, the thickness of the skin, and the skin's relative vascularization are likely to influence absorption kinetics. Skin thickness and vascularization often differ between skin regions in individual frogs and between amphibian species (Toledo and Jared 1993; Fox 1994; Young et al. 2005). Because the skin is integral to maintaining fluid balance in frogs, differences in thickness and vascularization between species correlate with habitat type (Roth 1973; Toledo and Jared 1993; Young et al. 2005). It is therefore likely that transdermal pharmacokinetics in frog skin will also differ, depending on the skin region and the frog species' primary habitat. However, although there are several studies that report outcomes for transdermal therapy in frogs (for review, see Llewelyn et al. 2016), most assess treatment success, with few transdermal pharmacokinetic studies. Further, studies investigating the effects of agricultural chemical exposure in frogs often focus on aquatic species or juvenile animals, with few studies in adult terrestrial frogs (for review, see Brühl et al. 2011). Finally, pharmacokinetic studies rarely investigate multiple skin regions or differences between species. These limitations have resulted in incomplete knowledge of transdermal pharmacokinetics in frogs.

Two recent studies (Kaufmann and Dohmen 2016; Llewelyn et al. 2018) directly investigated the in vitro transdermal kinetics of various standard chemicals in isolated frog skin using static diffusion cells. Each study investigated a species from a different habitat: Kaufmann and Dohmen (2016) conducted investigations in the aquatic frog *Xenopus laevis*, whereas Llewelyn et al. (2018) used the arboreal frog *Litoria caerulea*. Skin morphology in these species differs substantially, with *X. laevis* demonstrating a thicker skin overall with reduced vascularization across the entire body (Roth 1973) and *L. caerulea* having a relatively thick dorsum compared to its thin and highly vascularized ventral skin (personal observation). Absorption rates reported in these studies reflected these physiological differences in skin structure, with significantly higher flux of all chemicals through the ventral skin compared with the dorsum in *L. caerulea* (Llewelyn et al. 2018). A slight increase in ventral flux was also reported in *X. laevis* (Kaufmann and Dohmen 2016); however, the magnitude of the difference was far less than that reported in *L. caerulea*, being no more than a 2.3-fold increase for testosterone, compared to a 33-fold increase between regions seen for ventral versus dorsal flux of caffeine in *L. caerulea* (Llewelyn et al. 2018).

Terrestrial frog skin is structurally different from both aquatic and arboreal frog species. Typically, the outer epidermis of terrestrial species from arid locations is more keratinized and compact compared with that of aquatic species (Bani 1966; Toledo and Jared 1993), and although many terrestrial species have a highly vascularized pelvic region similar to arboreal species (McClanahan and Baldwin 1969; Christensen 1974; Toledo and Jared 1993), the difference in skin thickness between the ventral and dorsal skin in terrestrial species is often negligible (Schwinger et al. 2001; Willens et al. 2006). Interestingly, many terrestrial toads also have microscopic channels on the ventral and lateral skin surfaces, which act under capillary action to draw water from the pelvic region to the dorsum (Lillywhite and Licht 1974; de Brito-Gitirana and Azevedo 2005; Felsemburgh et al. 2009);

when present in arboreal species, these channels appear only to cover the ventral pelvic skin (Toledo and Jared 1993; Goniakowska-Witalińska and Kubiczek 1998).

Although a diverse range of chemicals have been reported to be systemically absorbed through the skin of adult terrestrial toad species, including high–molecular weight hormones and other chemicals with a range of relative lipophilicities (Table 1), few of these studies represent pharmacokinetic investigations. Indeed, the only study reporting in vitro transdermal pharmacokinetic data is by Willens et al. (2006), who investigated the in vitro percutaneous absorption of malathion, a moderately lipophilic (log*P* = 2.36) organophosphate insecticide, through dorsal and ventral skin of the terrestrial toad *Rhinella marina* (formerly *Bufo marinus*).

The present study expands on the extant knowledge of transdermal pharmacokinetics in amphibians by investigating the transdermal pharmacokinetics for 3 model chemicals in the terrestrial toad *R. marina*. These toads are native to Central and South America but are also found in many Pacific countries (Easteal 1981). Owing to its widespread distribution and the extensive biological, ecological, and physiological research on this species, *R. marina* represents an appropriate candidate species on which to establish baseline transdermal pharmacokinetics for terrestrial frogs.

MATERIALS AND METHODS

Study animals

Adult male *R. marina* (cane toads), wild-caught in the Townsville region (Australia), were used in the present study. Seventeen toads, ranging from 49.7 to 179.9 g body weight (mean = 116.2 g), were used, with toads randomly allocated to one of 3 chemical treatments. Seven toads were utilized for benzoic acid studies, and 5 were used for each of the remaining chemical treatments. Each animal was in good condition at capture and was euthanized

within 24 h of collection. All experimentation was completed in accordance with Animal Ethics approval A2222 from James Cook University, Australia.

Chemicals

Model chemicals were reagent-grade caffeine, American Chemical Society reagent-grade benzoic acid (both Sigma-Aldrich), and ≥98% ibuprofen (Sigma). Amphibian Ringer's solution (ARS) was prepared according to published methods (Wright and Whitaker 2001; 6.6 g/L sodium chloride, 0.15 g/L potassium chloride, 0.15 g/L calcium chloride, 0.2 g/L sodium bicarbonate), spiked with 2.75 g/L 2-hydroxypropyl-beta-cyclodextrin (HPβCD; Aldrich Chemistry) to assist solubilization in experiments using ibuprofen. Ethyl 3-aminobenzoate methanesulfonate solution (MS-222, 0.02 g/L; Aldrich Chemistry) was buffered to pH 7.3 with sodium bicarbonate. Methanol and acetonitrile were high-performance liquid chromatography (HPLC) grade (Fisher Chemicals and Thermo Fisher Scientific), formic acid was analytical grade (Thermo Fisher Scientific), and the water used in HPLC analyses was ultrapure (Milli-Q Integral; Millipore). All solutions were freshly prepared.

HPLC

The HPLC system was a Shimadzu Nexera-i LC-2040C 3D with photodiode-array detector. Postrun analysis was performed using LabSolutions 5.82 (Shimadzu). All HPLC methods used have been previously described and validated (Llewelyn et al. 2018; summarized in Table 2). All samples were run in triplicate.

Diffusion cell setup and skin preparation

Static Franz cell–type diffusion cells (Permegear), comprising a 1-mL donor chamber with a 9-mm orifice and a 5-mL receptor chamber, were used in these experiments. The donor chamber was filled with a saturated solution of one of the model chemicals (caffeine, benzoic acid, or ibuprofen) in ARS ± HPβCD ("infinite dose" conditions). Donor solutions

were prepared by placing an excess of chemical powder into a flask containing 10 mL (ARS for caffeine and benzoic acid experiments) or 50 mL (ARS ± HPβCD for ibuprofen experiments) of vehicle. The resultant mixture was then sonicated for 24 h at room temperature and centrifuged at 1500 *g* (4729 rpm) for 15 min, and the supernatant was used as the donor solution. The saturated solubility of each chemical in ARS (+ HPβCD for ibuprofen; Table 2) was determined by diluting the supernatant with vehicle to an appropriate level and analyzing the solution using the previously validated HPLC methods (Table 2).

The receptor fluid was ARS, with added HPβCD for the ibuprofen experiments. The receptor fluid was continually stirred with a magnetized stirrer bar and allowed to equilibrate in the diffusion cell for 30 min before the experiments commenced.

Toads were handled appropriately, to minimize damage to the skin. They were euthanized in a bath of buffered 0.02 g/L MS-222, and full-thickness ventral and dorsal skin samples were excised immediately. Following excision, each sample was rinsed in ARS and mounted on a diffusion cell with the internal skin surface facing the receptor chamber. Skin samples were visually inspected for damage prior to mounting on diffusion cells, and samples showing signs of damage were excluded from the study. Each animal provided 4 or 5 skin samples: dorsal and ventral pelvic samples were taken bilaterally along the truncal midline, whereas ventral thoracic samples were taken from the central truncal midline. In accordance with regulatory guidelines for dermal absorption testing (Organisation for Economic Co-operation and Development**<ZAQ;1>** 2004a, 2004b), a minimum of 4 replicates were obtained from multiple animals to allow for interindividual variability.

Determination of absorption kinetics

The methodology utilized in determining the cutaneous absorption kinetics in the present study has been described (Llewelyn et al. 2018). Briefly, 3 model chemicals—caffeine, benzoic acid, and ibuprofen—chemicals of similar molecular size (caffeine = 194.22

Da, benzoic acid = 122.13 Da, and ibuprofen = 206.28 Da) but differing relative lipophilicities (logP caffeine = –0.07, benzoic acid = 1.87, and ibuprofen = 3.97), were used. Each chemical was dissolved individually in ARS (plus 2.75 mg/mL HPβCD for ibuprofen) to achieve an infinite dose donor solution. After mounting skin samples on the Franz cell, 1 mL of donor solution was applied to the donor chamber. Samples (1 mL) were collected from the receptor chamber at t = 0, 30, 60, 90, 120, 150, 180, 240, 300, and 360 min for caffeine and benzoic acid and at t = 0, 30, 60, 90, 120, 180, 240, 300, 360, and 1440 min for ibuprofen. Fresh receptor fluid was added to maintain the receptor chamber volume following removal of each 1-mL sample. Samples were analyzed for chemical content using the validated HPLC method outlined in Table 2. Because this was an infinite-dose experimental design, mass balance was not performed (Organisation for Economic Co-operation and Development<**ZAQ;2**> 2004a, 2004b).

Cumulative absorption versus time plots were generated for each experiment, and curves were inspected for significant deviations suggestive of skin damage. Any results suggestive of skin damage were excluded from further analysis. Flux (micrograms per square centimeter per hour) was calculated for each sample from the steady-state slope of the cumulative absorption versus time plot.

Statistical analyses

Data were examined and analyzed using R (R Development Core Team 2016). The influence of chemical and skin region on flux through the skin was determined by fitting a linear mixed effects model using the nlme package (Pinheiro et al. 2017). The original model utilized chemical, skin region (dorsal, ventral pelvic, and ventral thoracic) and frog weight as fixed factors and included individual toad as a random effect so that each animal provided an individual unit of comparison. Model residuals showed significant variance in heterogeneity between skin regions, which was allowed for in the final model. Because toad weight did not

influence flux, it was removed from the final model. Following creation of the final model, the mean flux for each combination of chemical and skin region was analyzed using multiple pairwise comparisons and Tukey's post hoc test, with all pairwise comparisons implemented using the R package multcomp (Hothorn et al. 2008). Significance was determined for $\alpha < 0.05$ for all tests.

RESULTS

Cumulative absorption versus time plots showed that absorption for the chemicals decreased in the order caffeine > benzoic acid > ibuprofen through all skin regions (Figure 1). To further investigate these differences, flux for each chemical was calculated from the slope of the cumulative absorption versus time plots (Table 3) and subjected to statistical analysis. Initial analysis of the model found that both chemical ($F_{2,14} = 161.8546$, $p < 0.0001$) and skin region ($F_{2,47} = 4.4700$, $p < 0.0167$) significantly affected flux and that chemical flux differed by skin region ($F_{4,47} = 8.2158$, $p < 0.0001$; Figure 2). Multiple pairwise comparisons permitted further investigation of these effects. When comparing the flux of an individual chemical through different skin regions, no significant difference was found for flux between skin regions for either benzoic acid or ibuprofen; however, skin region did influence absorption of caffeine. The mean pelvic flux of caffeine (93.38 μg/cm^2/h) was significantly higher than mean flux through dorsal (78.76 μg/cm^2/h; $z = 6.216$, $p < 0.001$) and ventral thoracic (76.31 μg/cm^2/h; $z = 3.324$, $p = 0.0185$) skin regions. No significant difference in flux for caffeine was found between dorsal and ventral thoracic skin ($z = –0.503$, $p = 0.9998$).

Absorption through the skin is likely to be influenced by the relative lipophilicity of the chemical, so a plot was created examining the base 10 logarithm of flux (log-Flux) versus the logarithm of partition coefficient for dorsal, pelvic, and thoracic skin regions (Figure 3). For all skin regions, flux decreased with an increase in relative lipophilicity (log*P*). Interestingly, although flux appears relatively higher through the ventral pelvic skin

compared to the dorsum for the most hydrophilic chemical (caffeine), the more lipophilic chemicals exhibit a trend toward similar or lower ventral flux compared to dorsal flux. However, none of these effects were statistically significant (see Supplemental Data).

DISCUSSION

The in vitro absorption kinetics through the skin of the cane toad *R. marina* varied between chemicals, regardless of skin region. The most hydrophilic chemical (caffeine) demonstrated the most rapid flux, and a reduction in flux occurred with increasing relative lipophilicity of the applied chemical. Flux did not differ significantly between skin regions for either of the lipophilic chemicals; however, flux was significantly higher through the ventral pelvic skin than the dorsal or pelvic thoracic skin for caffeine, the most hydrophilic chemical. These differences can be attributed to known anatomical and physiological differences in skin thickness and vascularization between skin regions in *R. marina*, and knowledge of these trends will advise risk assessment following habitat contamination with xenobiotics.

Recent reviews (Brühl et al. 2011; Fryday and Thompson 2012; Weltje et al. 2018) have emphasized that when considering ecotoxicological impacts on terrestrial amphibians the transdermal route presents the most relevant exposure route. Terrestrial toads can be exposed to topical xenobiotics through a variety of mechanisms, including direct overspray of their habitat with agricultural chemicals; contamination of the soil and groundwater with previously sprayed chemicals; chemical release and runoff from agriculture and/or industry into the air, onto the soil, and into breeding pools; and the increasing problem of synthetic human medications in wastewater. Despite the pervasive presence of chemicals in the environment, dermal absorption studies in adult terrestrial amphibians are rare (Table 1), with fewer investigating absorption specifically in *R. marina*. Of the chemicals reported to be absorbed through the skin of *R. marina* having a systemic effect, the majority are of low

molecular weight and are mainly moderately lipophilic in nature. Further, of the studies in *R. marina*, only that of Willens et al. (2006) includes a pharmacokinetic investigation. In agreement with the present study, Willens et al. found no significant difference in the absorption rate between skin surfaces in *R. marina* for malathion, a moderately lipophilic chemical. In addition, the flux values reported for malathion (11.66 μg/cm^2/h dorsal and 11.28 μg/cm^2/h ventral "trunk") were similar to those determined in the present study for benzoic acid (12.96 and 9.02–11.32 μg/cm^2/h, respectively, for dorsal and ventral skin samples), a chemical with similar lipophilicity to malathion.

Unlike arboreal frogs, *R. marina* skin exhibits only slight regional differences in thickness, being marginally thinner on its ventral side (Schwinger et al. 2001; Willens et al. 2006). However, this difference in skin thickness is not statistically significant, and the number of cell layers in the stratum corneum (typically considered to be the primary barrier to percutaneous absorption) is the same for the dorsal and ventral skin regions (Willens et al. 2006). This similarity in skin thickness between skin regions is consistent with the present study's finding that skin region did not significantly influence flux of the more lipophilic chemicals. Because similar skin thickness between dorsal and ventral skin regions appears to be shared among other terrestrial toad species including *R. ornata* (Felsemburgh et al. 2009) and *Anaxyrus* (*Bufo*) *punctatus* (McClanahan and Baldwin 1969), the results of the present study may be extrapolated for other terrestrial frogs; and similarly, findings from transdermal studies in other terrestrial toad species may also be applicable to *R. marina*.

Although skin region did not significantly affect flux for either benzoic acid or ibuprofen, caffeine had significantly higher flux through the ventral pelvic skin compared with other skin regions. The likely explanation is that despite similar skin thickness between skin regions in *R. marina*, the pelvic region in frogs has a highly vascularized "drinking patch" with an enhanced water uptake function (Roth 1973). This "drinking patch" is present

in many frog species, particularly those not regularly associated with water, and has been shown to exhibit higher water flux than either thoracic or dorsal skin (McClanahan and Baldwin 1969; Bentley and Main 1972; Baldwin 1974). It is therefore likely that the increased water absorption from this region would facilitate concurrent absorption of hydrophilic chemicals such as caffeine. This finding is not unique; an increase in caffeine flux through the ventral pelvic skin is consistent with findings in both *L. caerulea* and *X. laevis*, with both studies reporting the highest and most variable flux through the ventral pelvic skin for caffeine (Kaufmann and Dohmen 2016; Llewelyn et al. 2018). However, the magnitude of the difference between regions differed between species, with the present study finding a 1.2-fold increase in flux, whereas a 1.5-fold increase was reported in *X. laevis* and a 33-fold increase in ventral flux compared to dorsal flux was reported in *L. caerulea*. Again, these differences can be explained when considering the skin morphology in these species. Like *R. marina*, *L. caerulea* also has a highly vascularized pelvic "drinking patch"; however, *L. caerulea* has markedly thinner skin on the ventral surface relative to its dorsum, which would inflate the difference in absorption between these skin regions. Conversely, the skin of *X. laevis* is only slightly thinner on its ventral surface, similar to *R. marina*, which supports the finding of similar flux levels between these regions.

Because lipophilicity is likely to be a primary influence on transdermal absorption through frog skin (Llewelyn et al. 2016), the chemicals included in the present study were selected to investigate the impact of increasing lipophilicity on transdermal absorption. In contrast to the findings in *L. caerulea*, which showed a parabolic relationship between log-Flux and log*P* for dorsal absorption and a linear relationship for ventral absorption (Llewelyn et al. 2018), the impact of lipophilicity on flux was consistent between skin regions in *R. marina*, demonstrating a decline with increasing lipophilicity. This finding corresponds with the findings of Kaufmann and Dohmen (2016), who reported a reduction in flux for

testosterone (log*P* = 3.3) compared with caffeine in *X. laevis*. A reduction in flux with increasing log*P* has also been reported in mammalian species (Dal Pozzo and Pastori 1996; Cross et al. 2003).

CONCLUSIONS

The permeability of frog skin provides an ideal route for absorption of both toxic and therapeutic chemicals. However, the lack of pharmacokinetic data on transdermal absorption in frogs means that the extent and rate of absorption of chemicals through the skin, and subsequent effects, cannot be accurately estimated. This is compounded by the potential impact on transdermal absorption by interspecies differences in skin thickness and vascularization. Because xenobiotics are pervasive in many frog habitats, understanding of the pharmacokinetics of transdermal absorption in these animals is central to risk assessment and environmental management. To this end, the present study investigated the baseline transdermal pharmacokinetic parameters for a series of model drugs in a terrestrial toad species. The finding that dorsal and ventral absorption rates are similar for moderate to highly lipophilic chemicals has implications for ecotoxicology and therapeutic use of chemicals in terrestrial toads. The majority of agricultural pesticides are of moderate to high lipophilicity (Finizio et al. 1997); thus, environmental contamination of and/or use of these chemicals in the toads' habitat is likely to result in significant absorption of these toxins, regardless of exposure surface or application method. However, the majority of therapeutic chemicals also have relative lipophilicity in this range, and these chemicals may be used in this species without regard to site of application. Determining these transdermal absorption parameters in frog species of differing skin physiology is essential to predict the rate and extent of xenobiotic absorption through frog skin, thereby informing risk analysis following exposure to toxic chemicals, environmental management in regard to habitat contamination with xenobiotics, and therapeutic management of disease in frogs.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4302.

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Data Accessibility—Please contact the corresponding author for access to data associated with the present study (tori.llewelyn@jcu.edu.au).

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Figure 1. Mean cumulative absorption versus time for 3 model chemicals through (**A**) dorsal, (**B**) ventral thoracic, and (**C**) ventral pelvic skin from adult *Rhinella marina*. Error bars show standard error.

Figure 2. Boxplots of flux for 3 model chemicals through dorsal, ventral pelvic, and ventral thoracic skin samples from adult male *Rhinella marina*. Lowercase letters indicate significantly different flux values ($p < 0.05$). Number of skin samples indicated. D = dorsal; P = ventral pelvic; T = ventral thoracic.

Figure 3. Logarithm of flux versus logarithm of partition coefficient through dorsal, ventral pelvic, and ventral thoracic skin samples from adult *Rhinella marina*. D = dorsal; P = ventral pelvic; T = ventral thoracic.

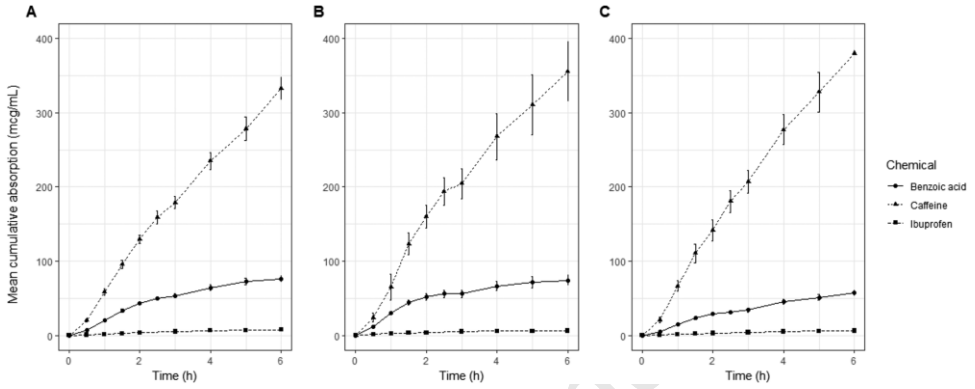

Figure 1

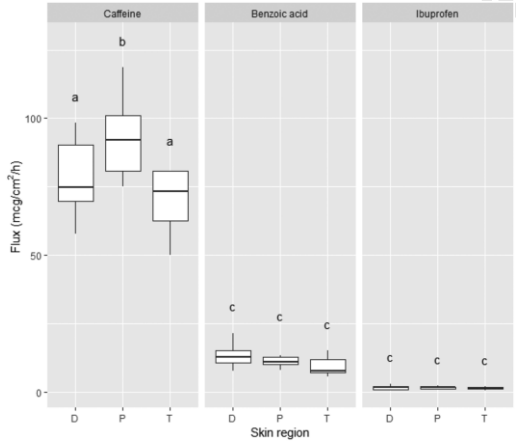

Figure 2

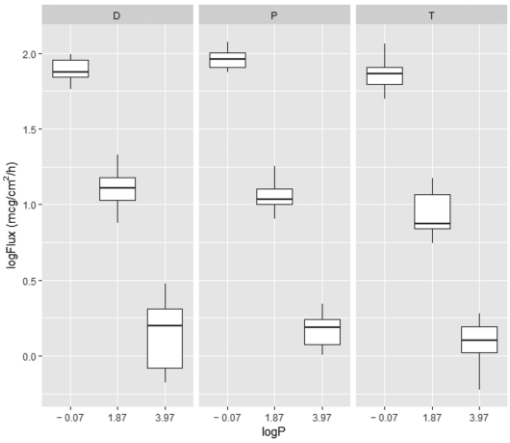

Figure 3