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Comparative abundance of cutaneous bacteria in Central European amphibians

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Abstract. The composition of cutaneous (skin-associated) bacterial communities of amphibians has been intensively studied in light of the potential of some of these commensal bacterial taxa to mitigate infection with the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). However, surprisingly, the absolute densities in which these bacteria occur on the skin are only poorly known. We here combine quantification of bacterial 16SrDNA copies from skin swabs by quantitative PCR with counts from scanning electron microscopy (SEM) images to assess and compare bacterial abundances on the skin of various central European amphibians. We focus on the fire salamander (*Salamandra salamandra*) which is severely threatened by the spread of *Bsal*. Based on counts from SEM pictures of selected skin parts, local densities of ~43,000 bacteria per mm^2 were ascertained in frogs, although the data are insufficient to understand whether these values apply to the whole body surface. Bacterial densities are doubtless much lower in fire salamanders in which we observed almost no bacteria in SEM examination. From qPCR data, we find: (i) statistically relevant differences in bacterial abundances among species, with the lowest abundances in terrestrial salamanders and the highest abundances in toads; (ii) higher bacterial loads in captive compared to wild fire salamanders, and much higher loads in *Bsal*-infected captive specimens, in agreement with the hypothesis that *Bsal*-infection leads to blooming of opportunistic bacteria that may cause secondary infection; (iii) an only weak decrease of bacterial load after repeated swabbing, in agreement with the hypothesis that skin swabs capture only a part of the bacteria of the swabbed skin surface. We discuss the multiple sources of uncertainty in absolute estimates of abundances of cutaneous bacteria and suggest further research to clarify and reduce these uncertainties.

Key words. Amphibia, Anura, Caudata, *Batrachochytrium salamandrivorans*, qPCR, scanning electron microscopy, skin microbiome, skin microbiota, bacterial abundance.

Introduction

Animals harbour diverse and individual-rich bacterial communities that play a crucial role for the host's physiology and health (McFall-Ngai et al. 2013). The complete microbial associates residing in and on a host's body are often referred to as *microbiota,* while the term *microbiome* usually refers to their joint genomes (Ley et al. 2008).

The role of the gut microbiota for physiology and health of animal hosts has been the subject of numerous studies (Lynch & Pedersen 2016, Gomaa 2020). In the intestinal tract, bacteria and other microorganisms play a crucial role in digestion, and especially herbivorous animals harbour numerous specialized symbiotic microbes in their intestine. A human body is estimated to include ~10^{13}–10^{14} microbial cells, with a ratio of around 1:1 microbial cells to human cells. The human colon alone has 3.8 × 10^{13} total bacterial cells, making it the organ with the highest density of microbes (Sender et al. 2016). In strictly herbivorous mammals, the number has been estimated at 10^{10}–10^{11} cells per ml in the rumen (e.g., Harfoot & Hazlewood 1988), similar to the human gut (Sender et al. 2016, Moran et al. 2019). For cellulose-digesting herbivorous insects such as termites, estimates of individual bacteria per gram of gut content are around 10^{7}–10^{11}, whereas for other insects such as caterpillars, flies, bees, or ants, values of 10^{4}–10^{10} have been reported (Moran et al. 2019).

Besides the intestinal microbiota, other animal-associated bacterial communities can also be of importance as it is obvious from the importance of particular bacterial species in the oral and the vaginal microbiota on the host's health (Lamont et al. 2018, Chen et al. 2021). A further important organ hosting diverse bacterial communities is the skin. In humans, despite being stable over time in indi-

viduals (Byrd et al. 2018), the cutaneous (= skin-associated) microbiome is strongly influenced by the environment (Boxberger et al. 2021). Furthermore, numerous studies in amphibians have shown that it is more strongly dependent from environmental reservoirs than the gut microbiome (e.g., Loudon et al. 2014, Bletz et al. 2016, Sabino-Pinto et al. 2017, Kueneman et al. 2019, Woodhams et al. 2020). Yet, the cutaneous bacterial and fungal communities can be of extreme importance for an organism, contributing to the skin's immune function and role as an initial barrier to pathogens, as it is known in humans (Sanford & Gallo 2013, Byrd et al. 2018), amphibians (e.g., Bletz et al. 2013, Rebollar et al. 2020, Christian et al. 2021), and – to some degree – in fishes (Takeuchi et al. 2021).

In amphibians, the health relevance of the cutaneous microbiota is particularly obvious in the context of emerging fungal diseases that affect mostly the skin. The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has caused dramatic declines in amphibians at a global scale (e.g., Scheele et al. 2019), and another chytrid species, *B. salamandrivorans* (*Bsal*), is ravaging European populations of salamanders after its likely introduction from Asia (Martel et al. 2014, Stegen et al. 2017, O'Hanlon et al. 2018). *Bsal* outbreaks in Europe are currently known from several areas in the Netherlands, Belgium, and Germany (Spitzen-van der Sluijs et al. 2016, Lötters et al. 2020), as well as one isolated case from Spain (Martel et al. 2020). In Central Europe, the most affected species is the fire salamander, *Salamandra salamandra*, which has undergone severe *Bsal*-associated population declines and local extinctions (Martel et al. 2013, Spitzen-van der Sluijs 2016, Lötters et al. 2020, Schulz et al. 2020). *Bsal* infects salamanders by invading keratinized parts of the skin, causing erosions and ulcerations (Martel et al. 2013). Furthermore, histological examination provided evidence that *Bsal* infection is often accompanied by bacterial overgrowth of the skin (Martel et al. 2013), and Bletz et al. (2018) showed that it leads to subtle changes in the skin microbiome which are associated with septicaemic events. Also for European newts, Bates et al. (2019) found that *Bsal* infection alters the composition of skin bacterial communities.

An important aspect in the discussion of strategies to mitigate chytrid fungi is the interaction of these pathogens with other skin-associated microorganisms (e.g., Woodhams et al. 2011, Thomas et al. 2019). The effect of the cutaneous bacteriome, and of particular bacterial species, against chytrid infection has been shown in numerous correlative and experimental studies (e.g., Harris et al. 2006, Lauer et al. 2007, Becker et al. 2009, Becker et al. 2015, Muletz-Wolz et al. 2017), suggesting probiotic mitigation strategies via bioaugmentation of beneficial, chytrid-inhibiting bacteria (Bletz et al. 2013). In the *Bsal*-susceptible fire salamander, the composition of the cutaneous microbiome has been investigated by Bletz et al. (2016) and Sanchez et al. (2017) and, as in other amphibians, is dominated by representatives of Proteobacteria, with slight differences among different parts of the body, developmental stages, and habitats. Several bacteria with *Bsal*-mitigating properties have been isolated from the fire salamander skin (Bletz et al. 2018). These effects could be experimentally shown in vivo, when the respective bacteria were applied at high concentrations, leading to the hypothesis that under natural conditions, the *Bsal*-mitigation effect of the microbiome might not be effective due to an insufficient absolute abundance of the relevant bacteria (Bletz et al. 2018).

While many recent studies have targeted the composition of the cutaneous microbiota of amphibians, it is striking that almost no data is available on the absolute abundances of bacteria on the skin. However, at which density bacteria inhabit a certain surface might be important for the function of a microbiome. Recent research emphasizes the importance of cell-to-cell communication mechanisms among conspecific as well as heterospecific bacteria, so-called quorum sensing, which usually coordinates gene expression patterns depending on cell density (Williams et al. 2007). Quorum sensing relies on diffusible secreted signals that trigger changes in bacterial behaviour when the population density reaches a critical value (Abisado et al. 2018). Although little is known about these mechanisms in cutaneous microbiota, there is evidence for their relevance in protection against epidermal injury in humans (Williams et al. 2019). Bacteria-bacteria interactions are also related to fungal resistance in cnidarians (Fraune et al. 2014), and quorum sensing genes have been identified in the cutaneous microbiome of the Neotropical frog *Craugastor fitzingeri* (Rebollar et al. 2018).

The total abundance or density of microbial cells on the skin of animals is, in general, poorly known. For humans, the density of cutaneous bacteria has been reported as 10^8 cells per mm² (Sender et al. 2016) based on culture-based assays performed more than 30 years ago (Leyden et al. 1987); values per mm² ranged from 1.7×10^5 in dry skin regions such as the extremities, to 1.4×10^9 in the healthy foot interspace, increasing to up to 1.4×10^{10} in the foot interspace of patients with Dermatophytosis Complex. More recent estimates of cutaneous bacteriome density vary from an average of 5.8×10^7 bacteria per mm² on the sole of human feet (Verhulst et al. 2011) to 10^4–10^6 cells per mm² on the skin of fishes (Austin 2006), which agrees with estimates of 10^4–10^5 cells per mm² on leaf surface in plants (Lindow & Brandl 2003). In amphibians, Cramp et al. (2014) found densities of ~10^3–10^4 colony-forming (culturable) bacteria per mm² on dorsal and ventral skin of the tree frog, *Litoria caerulea*, the lowest values corresponding to individuals directly after skin sloughing. In fire salamanders, Bletz et al. (2018) used quantitative PCR (qPCR) assays from skin swabs and found an average abundance of 3.5×10^4 rRNA copies per swab, which they translated into a tentative estimate of 5.7×10^3 rRNA copies per mm² of skin.

The composition of the bacterial microbiome is usually analysed by PCR-based DNA metabarcoding of the bacterial 16S rRNA gene (e.g., Caporaso et al. 2011, Pollock et al. 2018), which without normalization via spike-in standards (e.g., Stämmler et al. 2016, Tourlousse et al. 2017, Zemb et al. 2020) produces only relative, but not absolute abundance data. Here, we try to close this gap of knowl-

edge and provide comparative data on bacterial abundance on the skin of selected Central European amphibians obtained by qPCR based assays from skin swabs with a special focus on the fire salamander, and compare these data with direct bacterial counts obtained from scanning electron microscopy (SEM) of skin samples of the same species. It is our goal to provide preliminary data to develop initial hypotheses on (i) differences in bacterial abundance among amphibian species, and possible correlations with habitat and skin texture, (ii) effects of multiple swabbing on recovered bacterial abundance, and (iii) the effect of severe *Bsal* infection on bacterial abundances in fire salamanders.

Materials and methods

Sampling

Samples (skin swabs of adult amphibians) were collected opportunistically in several sites in Germany in 2019–2021. Fire salamanders (*Salamandra salamandra*) were primarily swabbed at the Sterkrader Wald in Essen (decimal geographical coordinates: 51.5431, 6.8356), a site where *Bsal* was not present at the time of sampling (Schulz et al. 2020). Captive fire salamanders kept in quarantine (both *Bsal* positive and negative, see below) originated from the Kruppwald, Essen (51.4046, 6.9948). Specimens of an introduced population of Italian cave salamanders (*Speleomantes italicus*) were swabbed near Holzminden in the Solling area (51.83, 9.46). Fire-bellied toads (*Bombina bombina*) were sampled at Kollau, Leipzig (51.4274, 12.6605). Specimens of the frogs *Pelophylax* kl. *esculentus*, *Rana dalmatina, R. temporaria, Bufo bufo* and the newts *Ichthyosaura alpestris, Lissotriton helveticus, L. vulgaris,* and *Triturus cristatus* were swabbed at two sites near Braunschweig: Elm (52.2172, 10.7453) and Kleiwiesen (52.3296, 10.5824).

For the quantification of cutaneous bacterial abundance, specimens were caught either by gloved hands or by dip nets. Each captured individual was held with a separate pair of gloves, rinsed with purified water (hereafter MQ water) to remove debris and transient microbes, and with ten up and down strokes on its ventral skin surface using a sterile MW113 swab (Medical Wire and Equipment, Corsham, UK). Swabs were stored separately in sterile vials and immediately transferred into a −20°C freezer, avoiding scenarios that could lead to bacterial overgrowth during transport (Anslan et al. 2021). Amphibians were returned to the ground or ponds immediately after the sampling of all individuals at a given site was completed.

Altogether, sampling resulted in 166 samples of eleven amphibian species. In detail, 13 *Bombina bombina* (Kollau), 28 *Bufo bufo* (Kleiwiesen, n = 26; Solling, n = 1, Wolfsburg, n = 1), eleven *Rana dalmatina* (Elm), six *R. temporaria* (Kleiwiesen), two *Lissotriton helveticus* (Elm), 26 *L. vulgaris* (Kleiwiesen, n = 25; Elm, n = 1), five *Ichthyosaura alpestris* (Elm), 42 *Pelophylax* kl. *esculentus* (Kleiwiesen), 47 *Salamandra salamandra* (Sterkrader Wald, wild, n = 26; Kruppwald, captive quarantine, n = 21), seven *Speleomantes italicus* (Solling) and 11 *Triturus cristatus* (Kleiwiesen) were sampled. All newt samples were taken during the aquatic reproduction phase, and all frog samples (*Rana, Pelophylax*) were taken from individuals captured in the water during the reproductive period. Toads (*B. bufo*) were sampled at two separate events: first, terrestrial specimens during the spring migration when their skin is partly muddy and many specimens are in the process of sloughing skin; and second, during their aquatic reproduction, when specimens have completed skin sloughing and are swimming in water, without any obvious mud or sand on their skin.

Furthermore, 24 individuals of *S. salamandra* from the Sterkrader Wald (all *Bsal*-negative) were subjected to multiple swabbing, i.e., the same individual was sampled five consecutive times (each time with ten up and down strokes). For twelve individuals, swabbing was carried out after rinsing with MQ water, for the other twelve individuals it was performed without any rinsing. One of the non-rinsed individuals was excluded from analysis, because it had unrealistically high bacterial loads at the first swabbing event of almost one order of magnitude higher, probably caused by mud or debris on this swab. For the final analysis, we therefore kept data of eleven non-rinsed and twelve rinsed individuals.

Samples were collected in October 2019 (*S. salamandra*), in October 2020 (*S. italicus*), and in March as well as April 2021 (*B. bufo, I. alpestris, L. helveticus, L. vulgaris, P.* kl. *esculentus., T. cristatus, R. dalmatina, R. temporaria*). Control samples were taken at almost every sampling event. Swabs of terrestrial microbial community samples were taken from dead wood and trees, and aquatic microbial community samples were taken from the pond water at the time of amphibian sampling.

DNA extraction and quantification of bacteria

The swabs were analysed in the laboratory of the Technische Universität Braunschweig (Braunschweig, Germany). Genomic DNA was extracted from the swabs using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's Animal Tissues protocol with pre-treatment for gram-positive bacteria. Incubation for the initial enzymatic lysis was extended to 1 h, and the temperature of the proteinase K lysis was increased to 70°C to increase DNA yield.

Bacteria were quantified using a SYBR Green qPCR assay and the universal bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). For the analysis, 5 µl iTaq Universal SYBR Green Supermix (BioRad), 0.3 µl of each primer, 2.4 µl MQ water was mixed with 2 µl of each sample. Primer concentration was 0.5 mM. qPCR conditions were 5 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 57°C, 30 s at 72°C (Tkacz et al. 2018). qPCR was performed on a CFX96 Real-Time PCR-System (Bio-Rad Laboratories Inc., Hercules, USA). Each sample was run in duplicate. Each qPCR plate contained two replicates of bacteria standards (10-

fold dilution scale from 4–400.000 16S copies for fire salamander samples; 10–1.000.000 for others) and two negative controls (MQ water). Raw data was analysed with the software CFX Manager v3.1 which, taking into account the standard curves and the quantification cycle (Cq), calculated the starting quantity (SQ) of 16S copies for the 2 µl of DNA extraction that was used for the qPCR reaction. We then: (i) calculated average SQ from the two replicates run per sample, (ii) subtracted from the sample's SQs the respective values obtained from DNA extraction negative controls run on the same qPCR plate, and (iii) multiplied the SQ values by 25 to account for the total amount of 50 µl DNA extraction yield per sample.

A table with all original qPCR data for the samples analysed has been deposited in the Zenodo repository (DOI: 10.5281/zenodo.6985186).

Scanning electron microscopy

To visualize cutaneous bacteria we used scanning electron microscopy as described before (Schulz et al. 2020). Briefly, skin fragments of three freshly killed amphibians (*I. alpestris, R. temporaria, S. salamandra*) were fixed with 5% formaldehyde and 2% glutaraldehyde overnight, washed twice with TE buffer, and dehydrated in a graded series of acetone (10, 30, 50, 70, and 90% on ice, and twice in 100% acetone at room temperature) for 20 minutes each. We applied critical-point drying with liquid CO_2, using a Balzers CPD 30 and gold-palladium sputter coating with a BalTec SCD500. Samples were examined with a field emission scanning electron microscope Merlin from Zeiss using both the HESE2 Everhart Thornley SE detector and the in-lens SE detector, and applying an acceleration voltage of 5 kV. Digital SEM images were made from representative skin sections with and without visible bacteria from each sample. These images were processed with Adobe Photoshop version 9.0 to slightly increase contrast for better visualization of bacteria, and to crop non-informative margins. We then used ImageJ version 1.53 (Schneider et al. 2012). Counts were performed on selected pictures with well-recognizable bacteria which might not be representative for the entire skin of the amphibian individuals studied. Since samples for SEM analysis were neither collected following a predefined nor a randomized design, our count data provide examples of encountered minimum and maximum numbers of visible bacteria per surface unit but cannot be used for statistical comparisons.

Statistical analyses

To compare bacterial (16S) abundance and Cq values among categories of samples, we used analyses of variance (ANOVAs) followed by Tukey's post-hoc tests, performed in Statistica v7.1 (Statsoft Inc.). Boxplots visualizing these differences were computed in JMP vs13.0 (SAS Institute Inc.).

To analyse the effect of rinsing and multiple swabbing on bacterial load, we used a Poisson-distributed Generalized Linear Mixed Model (GLMM) that is robust against non-normally distributed data, as implemented in JMP with the GLMM add-in (https://community.jmp.com/t5/JMP-Add-Ins/Generalized-Linear-Mixed-Model-Add-in/ta-p/284627). Specifically, the GLMM included as fixed effects: (1) MQ rinsing / not rinsing, and (2) Swabbing event 1–5 as ordinal variable; and as random effect the identification number of the individual salamander.

Results

Fire salamanders have low bacterial abundances compared to other terrestrial amphibians

Among 197 samples of wild amphibians, qPCR identified 16S loads per swab ranging from 1.43×10^4 (in an individual of *S. salamandra*) to 7.37×10^6 (in an individual of *B. bufo*), not counting two extreme outliers (one individual of *R. temporaria* with $1.1 0 \times 10^7$, and one individual of *L. vulgaris* with 7.62×10^6) (Fig. 1). Samples of the same species and locality spanned over two orders of magnitude, for instance between 1.43×10^4 and 3.38×10^6 in *S. salamandra* (n = 26), between 8.13×10^4 and 2.10×10^6 in *P.* kl. *esculentus* (n = 61), or between 1.87×10^5 and 7.37×10^6 in *B. bufo* (n = 28).

An ANOVA revealed a highly significant effect of species on bacterial load (log-transformed bacterial (16S) load values; DF = 10; F = 17.13; P < 0.0001). Tukey post-hoc tests found significant differences (P < 0.05) for the following pairwise comparisons (Supplementary Table S1): *B. bufo* significantly differed from all other species except for *L. helveticus; B. bombina* from *L. vulgaris, T. cristatus,* and *R. dalmatina; S. italicus* from all species except for *B. bombina, R. temporaria,* and *S. salamandra;* and *L. vulgaris* from *S. salamandra* and *P.* kl. *esculentus* (Supplementary Table S1). A graphical representation of the bacterial load values (Fig. 1) showed that the lowest average bacterial loads encountered corresponded to the two terrestrial salamander species (*S. italicus, S. salamandra*) and the (aquatic) fire-bellied toad *B. bombina*. Two other frogs sampled in their aquatic phase (*P.* kl. *esculentus, R. temporaria*) had rather low bacterial load values as well, while the values were higher in the four species of aquatic newts that were sampled on the same dates in the same habitats. The highest average value corresponded to the toad *B. bufo* that was sampled partly in the terrestrial and partly in the aquatic phase; no significant differences were found between terrestrial vs. aquatic phase of toads (t-test; t = -1.724; P = 9.097). In several species, outliers with unusually high values were detected (Fig. 1), possibly reflecting the presence of bacteria from remaining mud or particles on the skin that had not been removed by rinsing.

Scanning electron microscopy revealed the presence of bacteria on several samples of amphibian skin. The following counts represent examples of the bacterial abundances seen but might not be representative for the whole skin surfaces of the sampled individuals or species. In *I. alpes-*

tris, small agglomerations of bacteria could be observed irregularly in different areas of the skin, including the secretion pores of skin glands (Figs 2A, B; data do not allow to classify the glands as serous or mucous). The skin of the *R. temporaria* individual studied was dorsally covered by rather regularly spaced bacteria (Fig. 2C), which in some cases appeared to be embedded in the mucus layer (Fig. 2F). In one area with particularly well visible bacteria (Fig. 2D) we manually counted a total of approximately 320 bacteria on 7500 µm², resulting in a density of 0.043 bacteria per µm², equivalent to 4.3×10^4 per mm². In *B. bufo*, we observed a high variation of the density of cutaneous bacteria, both among and within the same individual. In some individuals, we observed very dense agglomerations of bacteria in areas of uneven skin that possibly correspond to minute skin folds in between skin tubercles (Figs 3A, B) while in others, large areas of skin had virtually no bacteria while densely packed groups of bacteria were present locally (Figs 3C, D). In several of these toads, we observed bacteria deeply embedded in or covered by a matrix which we interpret as mucus layer (Figs 3E, F). In contrast, SEM pictures of fire salamander skin in most cases revealed no bacteria (Figs 4A–C), similar to previous reports (Bletz et al. 2018); only a few bacteria, single or in small groups, showed up in a limited amount of images (Figs 4C, D).

Bsal-infected fire salamanders have strongly increased bacterial loads

We compared bacterial loads obtained for fire salamanders captured and swabbed in the wild (Sterkrader Wald), and specimens from Kruppwald taken into quarantine and kept for several weeks in captivity. This group of swabbed salamanders contained both *Bsal*-positive individuals, some of which in advanced stage of disease, and *Bsal*-negative

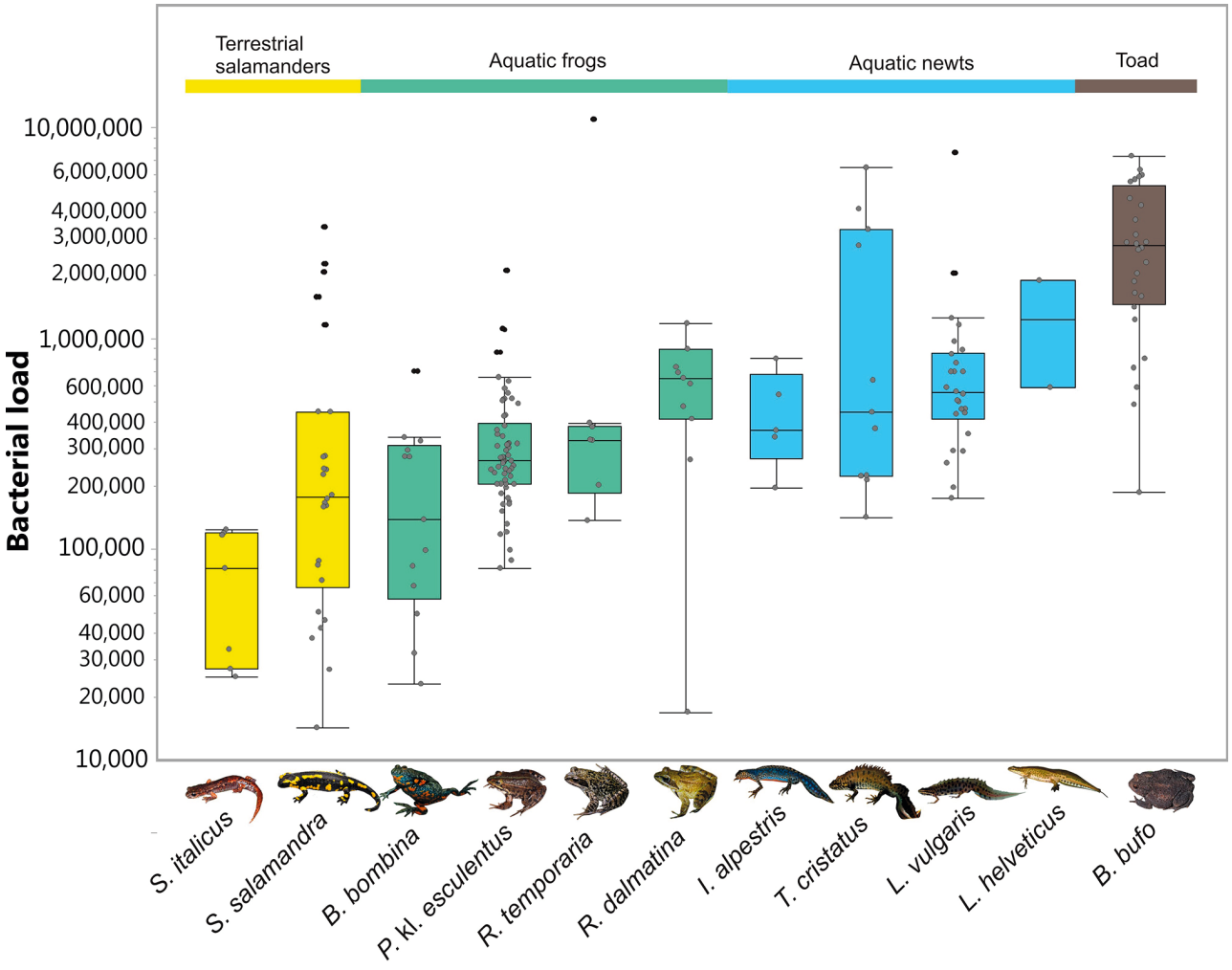

Figure 1. Diagram showing comparison of bacterial loads (number of 16S rDNA copies per skin swab) among amphibian species, determined using qPCR. All specimens were swabbed immediately upon capture in the wild and after rinsing with MQ. Note that bacterial load axis is logarithmic. The boxplot shows median (horizontal line), 25th and 75th quantiles (box), minimum and maximum (lines), and outliers (black dots). Dots are single data points. According to an ANOVA of the log-transformed values of this data set, the effect of species on bacterial loads is highly significant (DF = 10; F = 17.13; P < 0.0001); see Results for more detailed statistics.

healthy individuals. Due to amplification failure of standards in the qPCR plate containing the captive samples and lack of DNA template for repeats, we refrained from transforming the Cq values into values of bacterial abundance but preferred to statistically compare Cq values only. The three wild salamander samples on the same qPCR plate as the captive ones (squares in Fig. 5) had Cq values similar to those from other qPCR plates, justifying the combination of wild samples from various plates in the analysis. Differences in Cq values between the three categories (Fig. 5) were highly significant (ANOVA, df = 2, F = 63.1, P < 0.001), as were all pairwise comparisons among the categories (Tukey's post-hoc tests, P < 0.001). The highest Cq values (corresponding to lowest bacterial loads) corresponded to the samples taken from wild salamanders (median and minimum–maximum: 25.2; 23.0–27.3). Among the samples taken from captive (quarantined) specimens, those from *Bsal*-positive salamanders had lower Cq values (18.9; 16.6–21.6), and thus higher bacterial loads, than those taken from *Bsal*-negative salamanders (22.0; 16.5–25.8). The median Cq value of the *Bsal*-positive captive salamanders (18.9) was similar to that of *B. bufo* samples (18.7). However, the minimum Cq values of one *Bsal*-positive and one *Bsal*-negative salamander (the latter being the outlier in Fig. 5) of 16.5–16.6 were lower than the minimum value in *Bufo* (17.1; corresponding to 7.37×10^6), suggesting that these salamanders had bacterial loads possibly exceeding 10^7 RNA copies per swab.

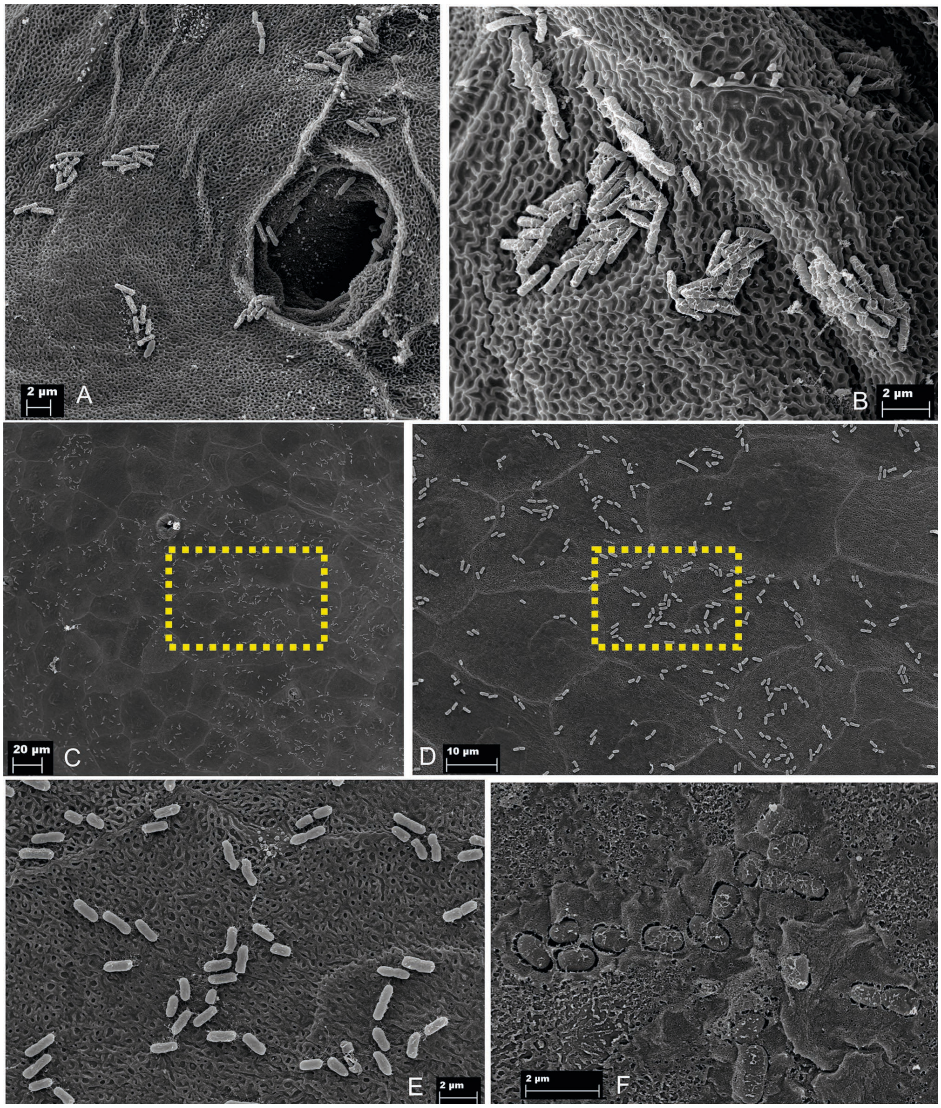

Figure 2. Scanning electron microscope (SEM) pictures of the dorsal skin of the newt *Ichthyosaura alpestris* (A, B) and the frog *Rana temporaria* (C–F) showing agglomerations of rod-shaped bacteria in the newt, partly inside of a skin gland pore, and large density of rather regularly spaced bacteria on the frog (C–E), as well as some bacteria partly embedded in what is likely skin secretion (F). Panels C, D and E show pictures taken at successively higher magnifications from the same location on one frog, the yellow boxes indicating the respective selection shown magnified on the next panel.

Weak effect of repeated swabbing on bacterial load

A GLMM for log-transformed bacterial load as response variable and individual salamander as random effect supported a weakly significant influence of swabbing event on bacterial load (DF = 1; DFDen = 85.7; F-ratio = 4.44; P = 0.0379). Visual inspection of these values (Fig. 6A) indicates a clear decreasing trend of bacterial load with successive swabbing in the non-rinsed salamanders where especially the first swabbing event yielded substantially higher bacterial loads than the subsequent swabbing events. This trend was not clearly recognizable in the salamanders that were rinsed before swabbing (Fig. 6B) which overall had lower bacterial loads, although the GLMM only revealed a tendency for significance of the rinsed/not rinsed variable (DF = 1; DFDen = 22.4; F-ratio = 3.22; P = 0.0863).

Discussion

At what densities do bacteria populate the skin of amphibians? This at first glance simple question proves to be difficult to answer, due to numerous methodological uncertainties. These extend from bacteria being removed during skin fixation and sample preparation for SEM to swabbing efficiency, variable 16S rDNA copies per bacterial genome, and presence of environmental DNA in qPCR-based approaches. In the following, we will discuss these uncertainties and highlight future studies necessary for an improved absolute quantification of the amphibian cutaneous microbiome.

The most immediate way of quantifying cutaneous bacteria is through microscopy. In particular, SEM, considering sample topology, has often been used to illustrate the presence or absence of bacteria on the skin surface of amphibians (e.g., Lauer et al. 2007, Bletz et al. 2018, Vaelli

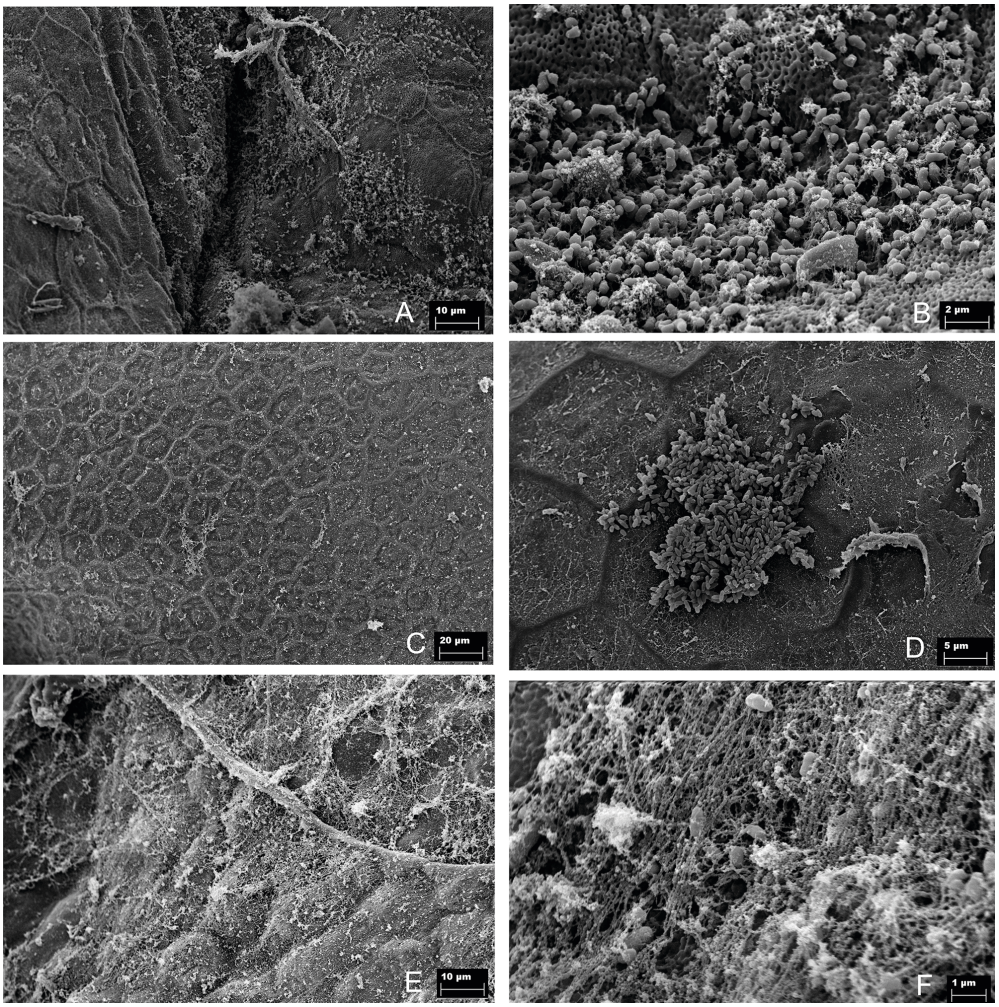

Figure 3. Scanning electron microscope (SEM) pictures of the dorsal skin of three individuals of *Bufo bufo,* showing high variation of bacterial density and locally high bacterial density in these amphibians. Each row of two panels corresponds to one toad individual. (A, B) Dense agglomerations of bacteria mixed with mucus matrix in a fold possibly corresponding to a separation of two skin tubercles. (C, D) Example of a specimen without bacteria on large parts of the skin (C) but agglomerations of numerous bacteria locally (D). (E, F) Example of specimen with skin densely covered with mucus and bacteria underneath or within this mucus layer.

et al. 2020). Our SEM pictures revealed that in many cases, these microorganisms appear to superficially colonize the amphibian skin (e.g., Fig. 2E), but we also observed bacteria more deeply embedded in the mucus (Fig. 2F). Therefore, on the one hand, bacteria and/or mucus might be washed away during the preparation of skin samples for SEM, although we performed all fixation steps with the utmost care (which still might be optimized using alternative fixation protocols, e.g. to better preserve anionic polysaccharides; Hammerschmidt & Rohde 2019). On the other hand, bacteria that are more deeply embedded in the mucus could be missed in the visual inspection of SEM pictures, although this might occur less frequently. Both issues could lead to an underestimation of SEM-based bacterial counts on skin samples. Here, the development of light microscopical (LM) techniques on integumental surfaces could certainly overcome the issue of losing bacteria. Fluorescent staining of DNA and 3D microscopy would allow to enumerate bacteria, or, if combined with specific probes as done in FISH, might also enable species identification. Clearing protocols in combination with light sheet microscopy might also be used to analyse the whole skin surface (e.g., Pinheiro et al. 2021, Subiran Adrados et al. 2021). However, FISH and light sheet microscopy will again include washing steps risking the loss of bacteria, and the same might be true for alternative fixation protocols for SEM. Furthermore, both SEM and high resolution LM examination require samples of freshly dead specimens which creates ethical and conservation concerns if larger sample sizes are to be studied. In the future, field microscopes (standalone or coupled to smartphones), applicable directly on a freshly collected tiny skin sample, or non-invasively on a living animal, might become an ideal tool to tackle these challenges – once such instruments allow resolutions down to the level of bacteria.

Determining the abundance of microbiota via qPCR allows processing of large sample sizes using a reliable and well-established technique. While this approach is power-

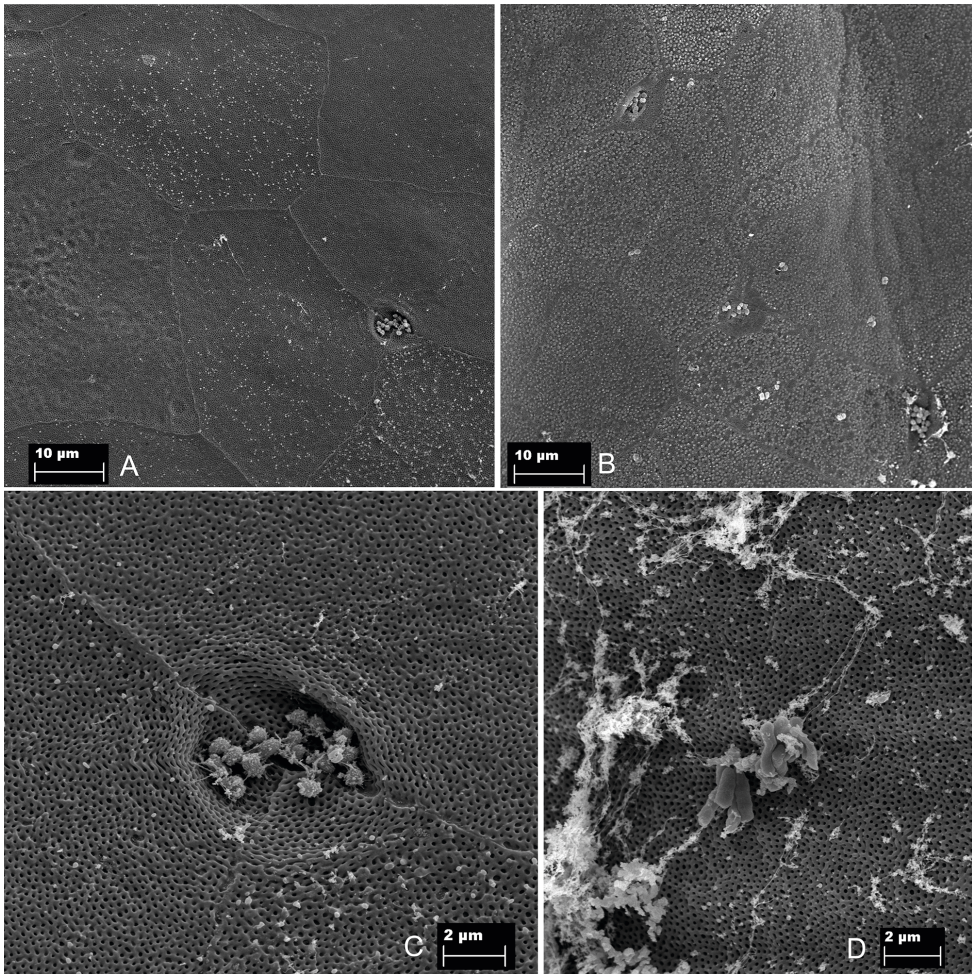

Figure 4. Scanning electron microscope (SEM) pictures with typical views of the dorsal skin of a fire salamander (*Salamandra salamandra*), with no recognizable bacteria (A–C). The small globules in the duct of the skin gland in C (zoomed in from A) likely represent secretion droplets but could also represent coccus-shaped bacteria. (D) shows one of the few instances where a small agglomeration of rod-shaped bacteria was observed on the fire salamander skin.

ful in comparing abundances among group of samples, e.g. among host species (Fig. 1), translating the obtained data into absolute densities of cutaneous bacteria is impeded by numerous uncertainties.

A minor issue that needs to be taken into account is that the approach quantifies bacteria indirectly via the number of sequences of the 16S rRNA gene of which bacterial genomes can contain between 1–15 copies, with an average of 2–3 copies in Proteobacteria, the group with highest frequency in amphibian cutaneous microbiomes (Větrovský & Baldrian 2013). The total number of living bacterial cells can thus be expected to be lower than the 16S copy values detected by qPCR, perhaps between 30–50% the number of 16S copies. A more substantial concern is that the qPCR approach will also detect DNA remains of dead bacteria or bacterial spores, which means that, at least theoretically, the number of living bacteria actively producing secondary metabolites and thus of relevance for the amphibian's immune system could be substantially lower than the number of detected gene copies. One option to overcome this latter issue would be the use of RNA instead of DNA as a template for the qPCR, as RNA decays more rapidly than DNA and therefore would allow quantifying of metabolically active bacteria only. In addition, a pre-treatment with propidium monoazide could be used to reduce the number of non-viable bacteria within the sample before DNA extraction (e.g., Kommerien et al. 2017).

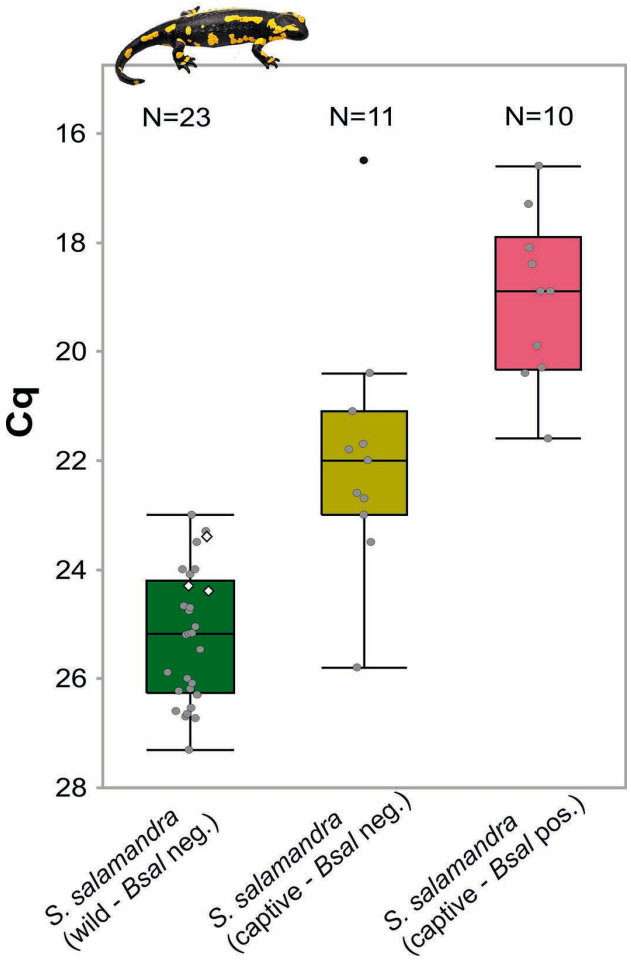

Figure 5. Diagram showing bacterial load determined from skin swabs of fire salamanders (*Salamandra salamandra*) from Sterkrader Wald (wild) and Kruppwald (captive) in Essen, Germany, using qPCR. Due to amplification failure of standards in the plate with captive samples (see text), quantification cycle (Cq) values rather than number of 16S rDNA copies are given. Note that Cq axis is reversed to better illustrate that low Cq values indicate high bacterial abundances. The boxplot shows median (horizontal line), 25th and 75th quantiles (box), minimum and maximum (lines), and one outlier (black dot). Dots are single data points. Three white rhomboids in the wild salamander category are samples analysed on the same qPCR run as the captive samples. All pairwise comparisons among categories were significantly different (Tukey's post-hoc tests after ANOVA, $P < 0.001$). All specimens were swabbed after rinsing with MQ.

A further, substantial uncertainty derives from the indirect quantification of cutaneous bacteria via swabbing. This approach relies on the assumption that intensive swabbing will capture most or all bacteria associated to the mucus layer of a particular skin surface. Contrary to that, our repeated-swabbing experiment (Fig. 6) suggests that this is not the case: repeated swabbing only partially depleted the population of cutaneous bacteria in fire salamanders, and even after four consecutive swabbing events, a substantial portion of these bacteria (or of their DNA) remain (Fig. 6). This means that the swabs contain only a fraction of those bacteria present on the swabbed skin surface, and that the actual density has to be higher than what could be estimated from the qPCR of a single swab.

A final uncertainty is also revealed from our multiple swabbing evidence, where a depletion of cutaneous bacteria can be observed in non-rinsed specimens but not in specimens rinsed before the initial swabbing event (Fig. 6); and from the many outliers having much higher-than-average bacterial loads in different amphibian species (Fig. 1). These findings confirm that transient bacteria, and especially bacteria attached to dirt particles or mud, can strongly bias the quantification of the cutaneous microbiome via swabbing approaches – a full removal of such biases via gentle rinsing might not always be possible (as in the outliers in Fig. 1).

Given these many uncertainties, at this point the attempt to estimate the bacterial density on the amphibian skin remains highly speculative. We here venture into this exercise, not to provide detailed values but to illustrate the associated problems more clearly. To provide minimum numbers, from our SEM results, we can confidently state that in frogs the bacterial density can reach at least ~43,000 per mm² (Fig. 2D) – which might be an underestimation if some bacteria had been removed during the sample fixation and preparation. In *B. bufo* we saw densely packed groups of bacteria, and bacteria partly embedded in or covered a mucus matrix, making it impossible to reliably count them from SEM pictures (Fig. 3), but the microscopic data are in accordance both with substantial individual differences among toads detected by qPCR, and with high

bacterial loads in some toads. We can also state that in fire salamanders, the bacterial density is probably substantially lower, as the respective SEM pictures revealed much fewer to no visible bacteria. For qPCR quantification, in our study we swabbed the entire ventral surface which amounts to ~2000 mm² in adult fire salamanders; obtaining an average load per swab of 5.35 x 10^5; this would yield densities of < 300 rDNA copies per mm². This corresponds to < 150 bacteria per mm² if assuming an average 2 rDNA copies per bacterial genome; the actual density might be higher because it is unlikely that we captured all bacteria with a single swab, or it might be lower since our swab might also have captured environmental bacterial DNA. Studying captive salamanders (which according to our results might have higher cutaneous bacterial abundances than wild individuals; Fig. 5), Bletz et al. (2018) used thorough swabbing of limited skin surfaces and estimated 5700 rDNA copies per mm². Thus, it overall seems that bacterial densities range from a few hundreds to a few thousands of bacteria per mm², while these values can be at least one order of magnitude higher based on both qPCR (Fig. 1) and SEM (Fig 2) data. We suggest that future studies should use very thorough swabbing of small and clearly delimited skin surfaces to obtain more reliable estimates, although it remains unlikely that a swab could take up all of the bacterial rDNA copies even from such a limited surface.

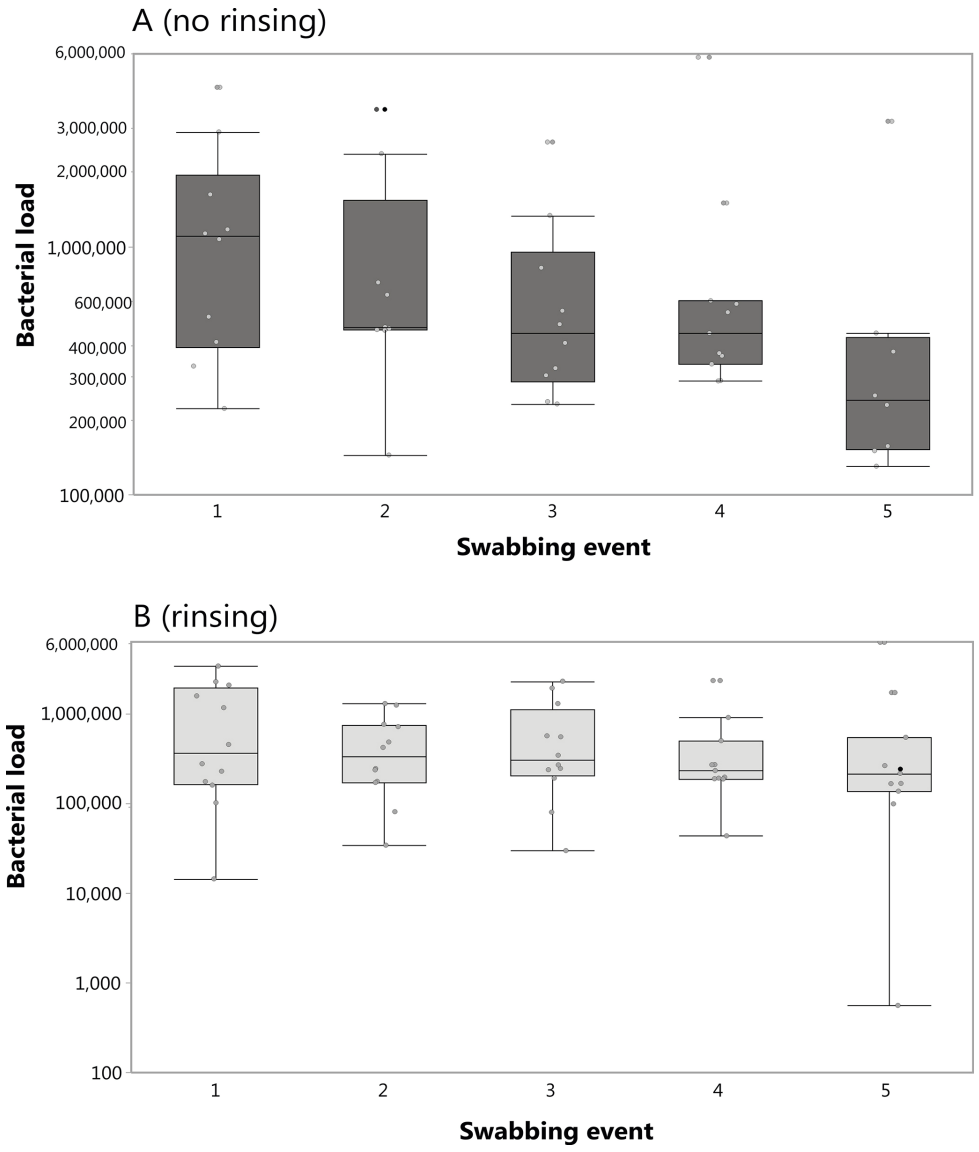

Figure 6. Diagrams showing bacterial load (number of 16S rDNA copies per skin swab) of fire salamanders (*Salamandra salamandra*) from Sterkrader Wald (Essen, Germany) determined using qPCR, for five consecutive swabbing events of each salamander. Data are shown for (A) eleven salamanders that were directly swabbed upon capture in the wild and (B) twelve salamanders that were rinsed with MQ water before the first swabbing event. Note that bacterial load axis is logarithmic. The boxplot shows median (horizontal line), 25th and 75th quantiles (box), minimum and maximum (lines), and outliers (black dot). Dots are single data points.

The composition of the cutaneous microbiome is influenced by numerous environmental factors, but is also influenced by the host. For instance, at the Kleiwiesen locality where many of our samples originated, drastic differences were found among microbiomes of co-occurring frogs and newts (Bletz et al. 2017), and in another case study, aquatic and terrestrial phases of the same newt species also differed substantially in relative bacterial abundances (Sabino-Pinto et al. 2017). Here, we demonstrate that these differences in microbiome species composition are also paralleled by differences in absolute abundances of cutaneous bacteria (Fig. 1). Although sample sizes were too low in many cases, *L. vulgaris* newts had significantly higher bacterial loads than *P. kl. esculentus* frogs that were caught in the same pond and swabbed on the same day. The highest average bacterial loads were found in *B. bufo* toads; we hypothesize that the highly expressed skin texture of these anurans may allow for a better attachment of bacteria, and perhaps simply leads to increased bacterial abundances due to overall higher colonizable surfaces, compared to the very smooth skin of terrestrial salamanders (Figs. 2–3). The strong differences observed among individuals of *B. bufo* (Figs. 1, 3) and other amphibians almost certainly are also related to skin sloughing which is known to reduce the number of cultivable cutaneous bacteria by up to 100% in amphibians (Meyer et al. 2012). This might exacerbate the risk of losing external bacteria in sample preparation procedures for SEM: these protocols include up to 10 washing steps, and it might be possible that the skin shortly after sloughing is smoother and with less fissures, with higher risk of bacteria and indeed great parts of the mucus layer being washed away.

Bletz et al. (2018) found average bacterial abundances of 1.4–6.4 x 10^4 16S rDNA copies per swab in four wild populations of fire salamanders. In our study, we found values of one order of magnitude higher in the Sterkrader Wald population (average 5.35 x 10^5) which we hypothesize might be related to differences in the amount of debris attached to the specimens; salamanders collected during heavy rainfall usually have very clean skin, while those just emerging from their shelters often have mud and soil particles on their skin that are not fully removed by rinsing. Six of the 26 swabs taken from salamanders in the wild had bacterial loads within the range of the average values of Bletz et al. (2018), suggesting the overall differences are not due to methodological differences or artefacts. In any case it remains true that in our study, the bacterial loads of fire salamanders are lower than those of many other terrestrial amphibians, and in part even lower than in amphibians in their aquatic phase when much less mud and debris with associated bacteria attach to the animal's skin.

While overall, fire salamanders appear to have a low bacterial density on their skin, our data demonstrate a substantial increase of bacterial abundances in individuals infected by *Bsal*. In amphibians, *Bd* infection is known to alter the composition of the cutaneous microbiome (e.g., Jani & Briggs 2014), and the same is true for *Bsal* infection where microbiome changes are associated with septicaemic events (Bletz et al. 2018). It is likely that these fungal pathogens influence the balance between the microbiota and the host's immune system. This might render commensals into opportunists or pathogens, as it has been found with disease and other stress scenarios in fishes (Kelly & Salinas 2017). The steep rise of cutaneous bacterial density in *Bsal*-infected salamanders detected in our samples is in agreement with a scenario where opportunistic taxa among the cutaneous or transient microbiota undergo fast blooming events on the damaged skin of their hosts, thereby causing secondary infections that might substantially contribute to the lethality of *Bsal*.

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Supplementary data

The following data are available online:

Supplementary document 1. Significance values from Tukey post-hoc tests from an ANOVA of log-transformed bacterial load values.