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RESEARCH ARTICLE

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Clinical and naturalistic substrates differ in bacterial communities and in their effects on skin microbiota in captive fire salamanders (*Salamandra salamandra*)

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ABSTRACT - The fire salamander (*Salamandra salamandra* and its relatives) is of increasing priority for ex situ conservation due to the spread of the fungal pathogen *Batrachochytrium salamandrivorans* in Europe. In captivity, the species may be maintained on a clinical paper-based or a naturalistic substrate, either of which has its own advantages and disadvantages. However, the impact of these two substrates on bacterial microbiotas within an enclosure and on the salamanders themselves is unknown. To investigate this, we maintained captive fire salamanders on either paper towels or a naturalistic substrate and quantified the culturable microbiotas of both substrates across the one-week lifespan of a paper towel and of the salamanders themselves over a six-month period. We found significant differences in the bacterial communities associated with the two substrates. Over a week-long period, there were major fluctuations in the community composition and abundance on paper towels while on the naturalistic substrate bacterial communities were relatively stable. The skin microbiota of salamanders were indistinguishable at the beginning of the study but after six months differed significantly between the two treatments, although the bacterial morphotypes present remained relatively similar compared with changes between substrates. These data show that husbandry protocols may have a strong influence on the culturable bacterial communities to which captive amphibians are exposed. Nevertheless, the animals were apparently able to maintain their own microbiota to a considerable degree. These findings should be borne in mind when determining husbandry protocols. Given the relative benefits of both types of enclosure, it is possible that a hybrid approach could be used whereby a small amount of naturalistic substrate is provided in a container within an otherwise clinical enclosure, to act as a bacterial reservoir.

INTRODUCTION

The establishment of ex situ populations of amphibians has become a core part of the strategy to counter global amphibian declines (Wren et al., 2015). Consequently, the number of amphibian species and individuals in captivity has greatly increased for conservation breeding (Tapley et al., 2015a), the investigation of disease dynamics (Burggren & Warburton, 2007), and the development of captive-care protocols (Antwis et al., 2014a; b; Michaels et al., 2014; 2015; Tapley et al., 2015b). In captivity, amphibian enclosures are arranged with a 'clinical' approach to husbandry in order to standardise methods, to improve biosecurity, and to ease maintenance workload. Such enclosures often have a paper towel substrate that is changed at regular intervals (~1-10 day) when they become soiled with amphibian waste and heavily contaminated with bacteria and fungi (Bishop et al., 2009; Garner et al., 2009; 2011; Retallick & Miera, 2007; Weinstein, 2009; Carver et al., 2010; Gahl et al., 2012; Ogilvy et al., 202; Ohmer et al., 2015; Venesky et al., 2015).

It has been shown previously that husbandry conditions significantly influence traits associated with amphibian health and fitness, in particular the composition and diversity of the skin microbiota (Antwis et al., 2014; Loudon et al., 2014; Michaels et al., 2014). Likewise environmental reservoirs are important in determining the microbiota of amphibian populations both in the wild (Fitzpatrick & Allison, 2014; Walker et al., 2014) and in captivity (Loudon et al., 2014). In nature, however, terrestrial amphibians live on a variety of complex mixtures of organic and inorganic substrates. Captive conditions should aim not only to promote health, welfare and ease of care for the animals being maintained but also to produce animals as similar as possible to wild individuals. This will ensure that experimental results are applicable to wild conditions and will generate a captive stock that has the best chances of survival after translocation to the field. Clinical substrates may act differently from natural substrates as reservoirs of bacteria (Loudon et al., 2014; Michaels et al., 2014), and this may have implications for the microbiota not only in the enclosure environment, but also on the skins of the animals maintained therein.

The fire salamander (*Salamandra salamandra*) is a widespread amphibian, distributed throughout most of continental Europe, and with close relatives in Europe (*S. atra*, *S. lanzai*, *S. corsica*) and in the Near East and North Africa (*Salamandra infraimmaculata* and *Salamandra algira* respectively). *Salamandra salamandra* is a complex of regionally restricted forms with radically differing morphology and ecology (Seidel & Gerhardt, 2017), and its conservation status is potentially better understood in this light. The recent discovery (Martel et al., 2013) and spread (Sabino-Pinto et al., 2015; Feldmeier et al., 2016) of the lethal

pathogen *Batrachochytrium salamandrivorans* (Bsal) in fire salamanders is a growing threat for the taxon, and plans for the creation of ex situ rescue populations of local forms of this species have been proposed and, in the case of the initial outbreak site for Bsal, enacted (Spitzen van der Sluijs et al., 2018). There are many approaches to maintaining fire salamanders in captivity (Seidel & Gerhardt, 2017), including the use of clinical paper-based substrates. These have been demonstrated to work well in terms of the clinical health of animals, and also offer the advantages of standardisation and biosecurity. However, their impacts on the skin microbiota of salamanders are currently unknown.

Here we quantify clinical and naturalistic substrates as environmental reservoirs of bacteria, and compare the effects of these husbandry conditions on a captive population of Spanish fire salamanders (*S. salamandra gallaica*) in order to inform husbandry practice for this species.

MATERIALS AND METHODS

Ethics Statement

All methods used in this study were non-invasive and did not require a UK Home Office Licence as they fell within best practice husbandry for this species. The University of Manchester Ethics Committee approved this study prior to commencement. Care was taken to ensure animals were not harmed during data collection, and individuals were monitored daily for signs of distress or injury, of which none were observed. Animals were rehomed after the completion of the study and no animals were destroyed as part of it.

Experimental design and animal husbandry

All experimental work was conducted in 2013 at the University of Manchester. Captive-bred (F2) *S. salamandra gallaica* were obtained as recently metamorphosed juveniles from a private breeder. Of eighteen salamanders used in test, the grandparents of eight were collected in the Sierra de Grandola, Portugal and ten from an unknown site in northern Spain. Animals from each site were allocated equally to each treatment by alternately selecting animals at random from each group. Animals were maintained individually in plastic containers (28 x 16.5 x 10 cm; Monkfield Nutrition, UK) with well-ventilated lids and access to a small water dish filled with aged tap water (GH <20mg/L, pH c. 6.5) changed weekly or when soiled. Refuges were provided in the form of overturned plastic plant saucers with doorways cut in the rim. 'Clinical' enclosures had a substrate of paper towels (blue paper towel hygiene rolls, Essential Supply Products, UK) dampened to saturation but not super-saturated with aged tap water. The towelling was spot cleaned daily for faecal material and then replaced weekly. 'Naturalistic' enclosures had a substrate consisting of coir coco-fibre (Wiggly Wigglers, UK), peat compost (B&Q, UK), rinsed silver sand (B&Q, UK), fine orchid bark (Monkfields Nutrition, UK) and crushed beech leaves in a 10:10:2:2:1 ratio. In nature, *S. salamandra* is found in woodland habitats, especially beech forest (Kuzmin et al., 2009) and this substrate was designed to mimic the leaf-mould substrate often found in this environment. All components of this mix were sterilised by autoclave prior to setting up enclosures but unlike the towelling it was not changed throughout the study, but was still subject to the manual removal of faecal material as it was produced.

Enclosures were arranged alternately in a climate controlled growth cabinet (Percival Scientific, Iowa, USA) with a 12:12 photoperiod, diurnal surface temperature of 17 °C and nocturnal surface temperature of 13 °C. All animals were fed every third day with black crickets (*Gryllus bimaculatus*) of an instar appropriate to the size of the salamander (cricket length approximately equal to the distance between the eyes), cricket guts were loaded for at least 24 hours on fresh fruit and vegetables and dusted externally with Nutrobal (Vetark, Winchester, UK) vitamin and mineral supplement immediately prior to being offered. Animals were also offered chopped earthworms (*Lumbricus terrestris*, Worms Direct, Maldon, UK) every fourth feed. All salamanders received the same prey species at any one feed and uneaten food items were removed after 24 hours.

Salamander growth rates

The mass of salamanders was measured at the beginning and end of the study (6 months) using Ascher AS2001 balances accurate to two decimal points.

Bacterial community culturing

Six months after the start of the study, bacterial communities associated with the clinical and natural substrates were characterised. A wet weight of 1 g of each substrate was collected using sterile tools, both immediately after fresh paper towels were placed in enclosures (day 1) and from soiled towels one week later (day 7). Substrate was placed in 10 ml of 1M $NaCl_2$ and vortexed vigorously for one minute. Substrate was left to settle for 30 seconds and then 1 ml of liquid pipetted off and used to construct serial dilutions to a concentration of 10-3 under sterile conditions. Bacterial communities from the salamanders were collected and cultured one week after animals were placed in experimental set-ups ('month 1'), and again six months later ('month 6'). The ventral region of the body was rinsed with sterile water and swabbed ~20 times (Michaels et al., 2014). Swabs were placed in 1 ml of 1M $NaCl_2$ to facilitate subsequent culturing methods, which were conducted under sterile conditions. Tubes containing swabs were vortexed to dissociate bacteria and then diluted ten-fold with 1M $NaCl_2$. For both substrate and salamander samples, dilutions of 100 and 10-1 were plated out on R2A agar media (Lab M Ltd., United Kingdom) and incubated at the same temperatures at which the salamanders were maintained. Bacterial colonies were grouped according to morphology and counted seven days after plating, after which negligible new colony growth was observed. Genetic sequencing was beyond the scope of the project, and so bacterial identification was not possible.

Data conversion and statistical analyses

Salamander body mass data were compared between treatment groups using repeated measures ANOVA in RStudio.

Bacterial counts were multiplied by the necessary dilution factors and averaged across the two dilutions for a given sample. All statistical analyses were conducted in RStudio. Differences in the total abundance of colony forming

units (CFUs) isolated from the two different substrates on day 1 and day 7 were analysed using a generalised linear model with 'tank' included as a random factor to account for the repeated sampling at two different time points. Differences in microbiota composition of the environmental substrates at days 1 and 7 were also analysed using an Adonis analysis with Bray-Curtis distance using raw count data and including 'tank' as a random factor, and these data were then visualised using nonmetric multidimensional scaling (NMDS).

The relative abundance of each bacterial morphotype in the overall community was calculated by dividing the number of colony forming units (CFUs) of a given bacterial morphotype by the total number of CFUs for each sample. In order to account for differences in bacterial abundance between the two treatment groups, and a high proportion of rare morphotypes in some samples, additional Adonis analyses were performed with this relative abundance data using Euclidean (as a distance measure) and Morisita–Horn (as a dissimilarity index) distances to test for differences between treatment groups at the two sampling points, with 'tank' included as a random factor.

Differences in overall bacterial community composition of salamanders were analysed separately at month 1 and month 6 using an Adonis analysis with Bray-Curtis distance using raw count data, and visualised using NMDS. Additionally, the relative abundance of each bacterial morphotype in the community was calculated for each individual at each sampling point and Adonis analyses with Euclidean and Morisita–Horn distances were used to test for differences between treatment groups. The microbiotas associated with salamanders six months after the start of the study were compared to bacterial communities associated with the substrate (using data from day 7) using an Adonis analysis with Bray-Curtis distance using raw count data and visualised using NMDS, and analysed using Adonis with Morisita–Horn and Euclidean distances for relative abundance data.

RESULTS

Mean body mass at the start of the study across treatment groups was 1.02 (± 0.41) g and mean mass at the end of the study (i.e. at 6 months) was 12.89 (±3.23) g. There was no significant difference between experimental groups of salamanders in the change in body mass according to treatment ($F_{1,85}$ = 1.191, p = 0.278).

Total abundance of bacterial communities isolates from the substrates was significantly affected by sampling time (X^2 = 25.680, d.f. = 1, p < 0.001), treatment group (X^2 = 19.963, d.f. = 1, p < 0.001), and their interaction (X^2 = 23.343, d.f. = 1, p < 0.001). Post hoc contrast analyses showed that the abundance of cultured bacteria was significantly higher for the clinical substrate at day 7 (p < 0.001 in all cases; Fig. 1). Bacterial community composition of the two substrates, based on raw bacterial abundance counts, were significantly different according to sampling time ($F_{1,32}$ = 24.788, p = 0.001), treatment group ($F_{1,32}$ = 26.415, p = 0.001) and their interaction ($F_{1,32}$ = 20.516, p = 0.001). The NMDS figure shows that culturable bacterial communities associated with naturalistic substrates remained stable over a one week period (Fig. 2; black shapes), but that communities associated with the clinical substrate were initially similar yet differentiated from those of the naturalistic substrate (Fig. 2; grey squares), and one week later these were significantly differentiated from naturalistic samples (Fig. 2; grey triangles).

The Adonis analyses of relative abundance data showed that for both distance measures there was a significant effect of sampling time (Morisita–Horn: $F_{1,32}$ = 14.762, p = 0.002; Euclidean: $F_{1,32}$ = 10.111, p = 0.001), treatment (Morisita–Horn: $F_{1,32}$ = 6.860, p = 0.014; Euclidean: $F_{1,32}$ = 7.531, p = 0.003), and their interaction (Morisita–Horn: $F_{1,32}$ = 38.166, p = 0.001; Euclidean: $F_{1,32}$ = 19.902, p = 0.001). Community composition and relative abundance of bacteria were stable over the one-week sampling period for the naturalistic substrate, but fluctuated massively for the clinical substrate, with one bacterial morphotype dominating the substrate by day 7 (Fig. 3A).

At month 1, the culturable bacterial community

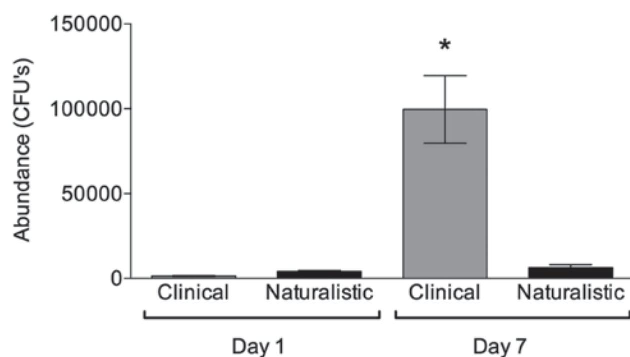

Figure 1. Total abundance of bacteria associated with clinical (paper towel) and naturalistic (organic material) substrates at day 1 and day 7. The * indicates a significantly (p < 0.001) different result to all others.

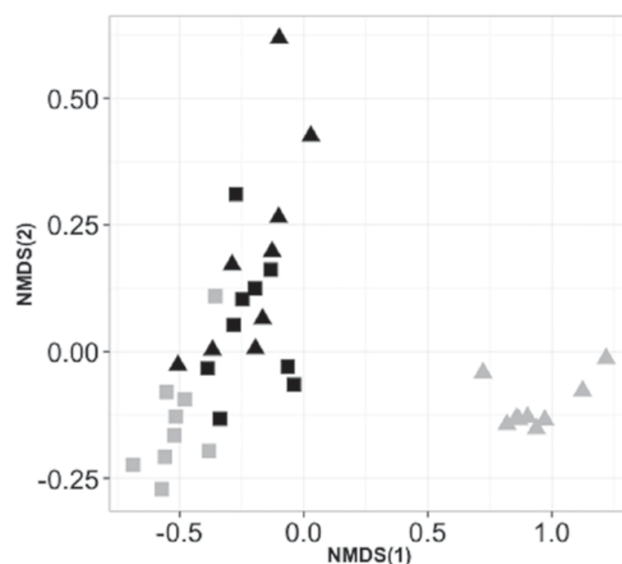

Figure 2. Non-metric multidimensional scaling figure depicting the bacterial community composition of salamander environments; naturalistic (black) and clinical (grey) environments at day 1 (squares) and day 7 (triangles), stress value = 0.04.

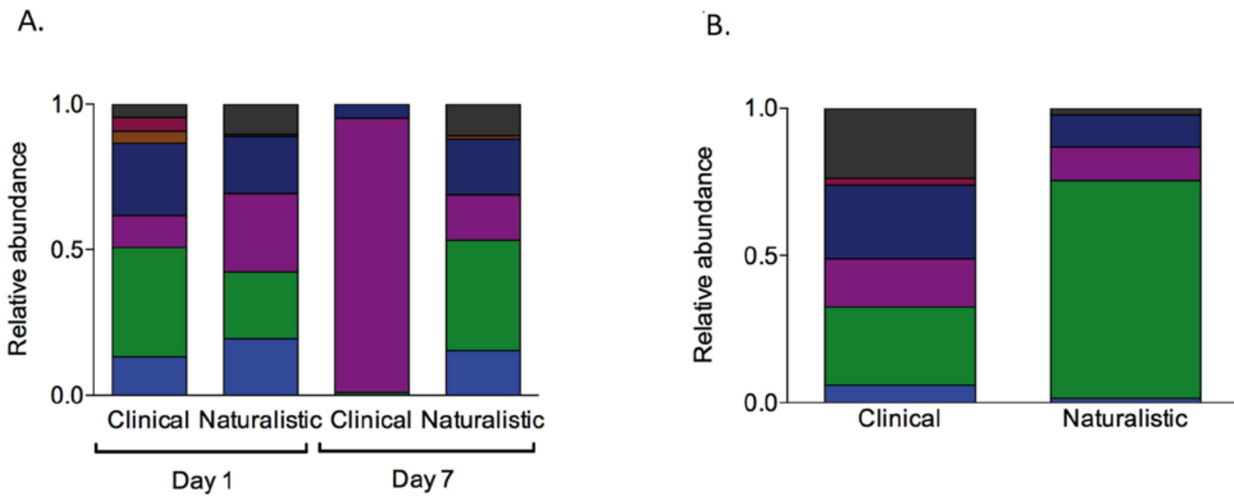

Figure 3. Relative abundance of culturable bacteria: **A.** Isolated from naturalistic and clinical substrates on which salamanders were maintained, **B.** Isolated from the skin of salamanders maintained in naturalistic and clinical environments 6 months after the beginning of the study. Different colours/shades represent different bacterial morphotypes, and colour/shade coding is conserved between the two figures.

composition of salamanders on the two different substrates were not significantly different based on total abundance counts ($F_{1,16}$ = 1.390, p = 0.186; Fig. 4A) or relative abundance data (Morisita–Horn: $F_{1,16}$ = 3.077, p = 0.055; Euclidean: $F_{1,16}$ = 1.755, p = 0.129). After six months on the two different substrates, overall community composition of salamander microbiotas were significantly differentiated based on raw abundance data ($F_{1,16}$ = 3.102, p = 0.021; Fig. 4B) and relative abundance data (Morisita–Horn: $F_{1,16}$ = 9.779, p = 0.002; Euclidean: $F_{1,16}$ = 10.030, p = 0.003). Salamanders maintained on the naturalistic substrate had only one dominant morphotype, with two intermediate morphotypes and a number of low abundance morphotypes (Fig. 3B), despite the naturalistic substrate exhibiting a relatively even community composition across bacterial morphotypes (Fig. 3A). Conversely, salamanders maintained on the fluctuating clinical substrate had a number of bacterial morphotypes with intermediate to high relative abundances, despite the substrate shifting from a relatively even representation of morphotypes on day 1, to the dominance of one bacterial morphotype by day 7.

Six months after the start of the study there were significant differences in the microbiotas associated with salamanders and substrates ($F_{1,32}$ = 10.285, r^2 = 0.150, p = 0.001), between the two treatment groups ($F_{1,32}$ = 13.965, r^2 = 0.203, p = 0.001), and a significant interaction between these two parameters ($F_{1,32}$ = 12.482, r^2 = 0.182, p = 0.001; Fig. 5) based on raw abundance counts of bacteria, which was also supported by the Adonis analyses of relative abundance data (p < 0.001 for all parameters and interactions using both Morisita-Horn and Euclidean distance measures). On the whole, bacterial communities associated with the skin of salamanders maintained on the naturalistic substrate (black circles; Fig. 5) closely resembled the bacterial community associated with the naturalistic substrate (black diamonds; Fig. 5). Bacterial communities associated with the skin of salamanders on the clinical substrate (grey circles; Fig. 5) were similar but slightly differentiated from those of the naturalistic substrate or salamanders maintained on the naturalistic substrate, whereas the bacterial community associated with the substrate in the clinical environment was vastly different from all others (grey diamonds; Fig. 5).

DISCUSSION

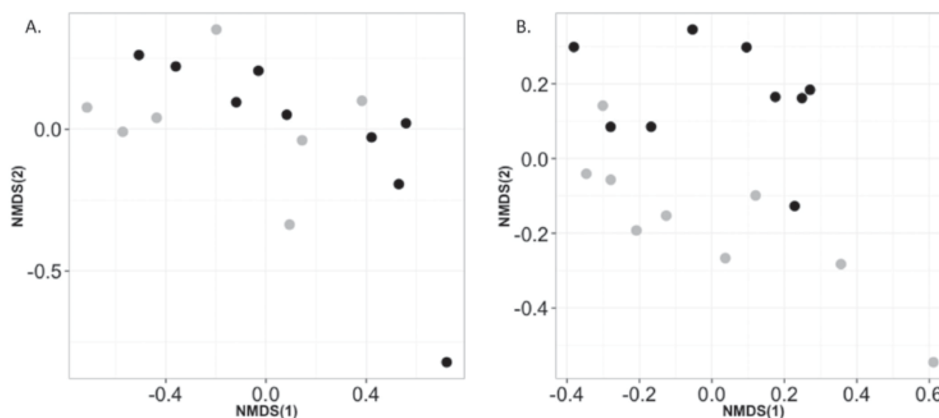

Figure 4. Non-metric multidimensional scaling figure depicting the bacterial community composition of salamanders associated with naturalistic (black) and clinical (grey) environments: **A.** At month 1, **B.** At month six (B), stress values = 0.06 and 0.10, respectively

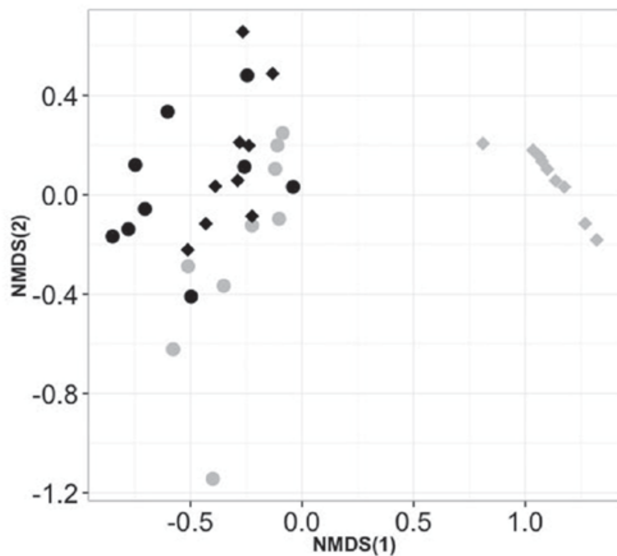

Figure 5. Non-metric multidimensional scaling figure depicting the bacterial community composition of salamanders and their environments (data represents samples collected on 'day 7' of sampling at 6 months after the start of the study). Black circles = salamanders in naturalistic environment; grey circles = salamanders in clinical environment; black diamonds = naturalistic substrate; grey diamonds = clinical substrate, stress value = 0.07

Our data show that naturalistic and clinical substrates in salamander enclosures are very different in terms of the culturable bacterial communities developing within them. Naturalistic substrates hold more diverse and stable bacterial communities, while the clinical substrate is much less stable and rapidly becomes dominated by a single morphotype. These findings reflect both the nature of the substrate and the maintenance regimes that they necessitate. The chemically and structurally complex nature of the naturalistic substrate likely allows for the regulation of a temporally stable microbiota, as well as providing a greater variety of environmental niches through variation in particle size and composition. Movement of bacteria through soil is often poor and this, along with highly localised co-evolution with bacteriophages, can lead to the development of a highly heterogeneous distribution of bacterial genotypes over a matter of centimeters (Vos et al., 2009). In addition, there is evidence that bacterial communities with a greater diversity exhibit higher temporal stability (Flores et al., 2014), and therefore such communities are, in effect, self-regulating.

Paper towels rapidly degrade and must be replaced frequently - in this case, weekly. This process resets the environment and therefore the associated bacterial community. This may have prevented the development of more complex microbiotas by interrupting succession and the development of complexity through competition; instead the faster reproducing morphotypes may have been favoured continuously. Moreover, the paper towel offered a more homogenous environment with a smaller range of niches for bacteria to grow, as well as probably also facilitating the movement of bacteria throughout the substrate - especially as when dampened there would be a continuous aqueous environment throughout the towel. These characteristics likely created an environment favouring lower diversity and reduced stability of microbiota.

As well as differing in stability and diversity of bacterial communities, the clinical substrate generated much higher peak abundances of bacteria during its weekly replacement cycle. The dominant bacterial morphotype became several orders of magnitude more abundant than total abundance of bacteria in the naturalistic substrate over the same timescale. Such blooms of bacteria may represent a health hazard for captive salamanders, as they may overwhelm immune responses (Seidel & Gerhardt, 2017). The combination of low diversity and high abundance of dominant bacteria may also allow invasion of pathogens more easily than a complex community. This result indicates the importance of frequent replacement of paper towel substrates.

We also demonstrated that the different substrates used for salamanders influenced the microbiotas of the animals themselves. Loudon et al. (2014) showed that organic matter was important for maintaining a "core" microbial community after moving adult salamanders (*Plethodon cinereus*) from the wild into captivity. Our data suggest that it is also important for maintaining the microbial community of captive bred animals that have not been exposed to microbiota in their natural habitat. Salamanders maintained on the naturalistic and clinical substrates also hosted significantly differentiated microbiotas. Four bacterial morphotypes occurred at much greater relative abundances on salamanders maintained on the clinical substrate compared to those maintained on the naturalistic substrate, for which only one bacterial morphotype predominated (Figures 3A and 3B). This pattern is the reverse of that found in the actual substrates, and may indicate that the fluctuating environment provided by the clinical substrate promoted diversity by creating temporal niches suited to different species. This situation differs from the substrate itself, as the salamander microbiome was not reset at the time of paper changes. However, despite these environmental fluctuations, salamanders reared on a clinical substrate maintained a broadly similar bacterial community in terms of the morphotypes present, if not the relative abundances of each bacterial type, compared to those reared on a naturalistic substrate (Figure 4). This supports the notion that amphibians regulate their skin microbiota, potentially through the production of anti-microbial peptides (Küng et al., 2014).

Species composition and community dynamics of microbiotas associated with *Salamandra* spp. are of particular interest given the recent emergence of a second lethal *Batrachochytrium* fungus (*B. salamandivorans*) in northern Europe, as a result of which massive population declines in this host species have been observed (Martel et al., 2013; 2014; Spitzen et al., 2013). However, the propensity for symbiotic bacteria of amphibians to mediate this pathogen is currently poorly understood.

In some other amphibian taxa, changes in microbiota caused by different husbandry approaches have correlated with differences in growth, such that conditions that promoted ostensibly more advantageous microbiotas also promoted growth (Michaels, et al., 2014). This is possibly

mediated by effects of husbandry on behaviour and environmental stressors, which may have implications for the health and fitness of amphibians. In the present study, however, we detected no effect of substrate on growth rates in salamanders, which suggests that while the substrate may be important for determining patterns in microbiota, it does not translate to other measures of health and fitness in this case. All salamanders grew at rapid rates, on average increasing mass 12-fold in 6 months, suggesting that all the animals were in good general health rather than poor health which in amphibians is often associated with limited growth.

The results presented here are based on morphotypic identification and culturing techniques, which do not allow inclusion of portions of the microbiome that are not culturable under the conditions used, and do not allow specific/strain identification of bacteria. Although molecular techniques are required to more fully characterise microbial communities, the data presented here offer convincing evidence that a given subset of the environmental and host-associated bacterial communities are susceptible to differences in substrate, and it is likely these differences are also seen in the non-culturable portion of the microbiota. Moreover, these data represent only a 6-month window into the effects of substrate on a species that can live for more than 30 years and it is as yet unclear if the differences detected here in skin microbiota might have longer-term implications for hosts not detected in this short period, or if additional effects of substrate on skin microbiota may emerge over a longer period.

We did not measure the accumulation of waste products in substrates. Salamanders produce nitrogenous waste, partially in the form of solid faeces, but also liquid waste containing ammonia. Despite the manual removal of faeces, waste products accumulate in substrates and can eventually become toxic (Seidel & Gerhardt, 2017). While frequent changing of clinical substrates as they degrade may influence environmental and skin microbiome microbial communities, it also avoids accumulation of toxic waste products. Given the relative benefits of both types of enclosure, it is possible that a hybrid approach could be used whereby a small amount of naturalistic substrate is provided in a container within an otherwise clinical enclosure, to act as a bacterial reservoir.

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