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Effects of aquatic and terrestrial habitats on the skin microbiome and growth rate of juvenile alpine newts *Ichthyosaura alpestris*

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Cutaneous bacterial communities can be crucial in modulating amphibian-pathogen interactions, but are highly sensitive to environmental conditions. Many amphibians, in particular salamandrid newts, may inhabit aquatic or terrestrial habitats after metamorphosis. These different conditions can alter the cutaneous bacterial communities of animals and so affect both the susceptibility of individuals to disease and their potential to transmit pathogens to others. Furthermore, different environments may influence the fitness of individuals through impacts on growth rates. I investigated the impact of aquatic and terrestrial environments on the cutaneous bacterial communities and growth rates in the alpine newt (*Icthyosaura alpestris*). This species is invasive in the UK and has been reported as carrier of amphibian pathogenic chytrid fungus. I show that aquatic animals, although growing faster, present less diverse communities, lacking in species that inhibit *Batrachochytrium dendrobatidis (Bd) in vitro*. My data suggest that aquatic and terrestrial phases in amphibians may influence their susceptibility to disease and I suggest that this likely impacts the way in which pathogens, especially *Bd*, spread in the environment.

Keywords: Amphibians; cutaneous bacterial communities; microbiomes; *Bd*; captivity, *Batrachochytrium dendrobatidis*, mutialistic bacteria, *ex situ* conservation

INTRODUCTION

Amphibians are globally threatened with extinction, with more than two-thirds of species in danger (Wren et al., 2015). Emerging infectious diseases, including chytridiomycoses caused by two different *Batrachochytrium* species (Longcore et al., 1999; Martel et al., 2013) have been identified as major and currently irreversible threats (Wren et al., 2015). In order to develop mitigation measures for these diseases, it is important to understand both the biology of the pathogen and that of the host. In particular, the importance of the host-associated skin microbiota is becoming increasingly clear (Jervis et al., 2021). Bacterial communities may inhibit pathogenic growth either by competition or through the active production of toxic compounds (Belden & Harris, 2007; Brucker et al., 2008a; b; Becker & Harris, 2010; Bates et al., 2018; Kueneman et al., 2019; Jervis et al., 2021). Therefore, the microbiome plays an important part in determining host-pathogen interactions, as well as the potential for amphibians to transport pathogens between and within habitats (Bletz et al., 2013).

Amphibians have complex life cycles often involving an aquatic larval phase followed by metamorphosis into a terrestrial form, which subsequently matures to breed. In salamander species, juveniles (post-metamorphosis) of some newt species may mature under aquatic (as metamorphs or paedomorphs) or terrestrial conditions. The skin microbiome is acquired from the environment to which animals are exposed (Fitzpatrick & Allison, 2014), as well as from other animals and influenced by abiotic factors (Ruthsatz et al., 2020), and even subtle differences in environmental parameters can influence the bacterial communities associated with amphibians, as well as the fitness of animals themselves (Loudon et al., 2013; Antwis et al., 2014a; Michaels et al., 2014; Kueneman et al., 2019). Furthermore, microbiome quality is frequently associated with amphibian health in the context of both disease processes (e.g. Bates et al., 2019; Jervis et al., 2021) and environmental impacts in captivity (e.g. Antwis et al., 2014a; b; Michaels et al., 2014) and in the wild (e.g. Kueneman et al., 2019). The radically different environments to which terrestrial and aquatic animals are exposed have the potential to profoundly influence cutaneous bacterial communities (Jervis et al., 2021).

Amphibian conservation is dependent, for many species, on *ex situ* assurance colonies (Wren et al., 2015), but it has previously been shown that symbiotic or mutualistic relationships between bacteria and the skin of amphibians are sensitive to the captive environment (Loudon et al., 2013; Antwis et al., 2014; Michaels et al., 2014; Becker et al., 2014; Passos et al., 2018; Harrison et al., 2019; Michaels & Preziosi, 2020). The effects of rearing environments and of aquatic and terrestrial phases on these communities in salamanders, however, are poorly known.

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Here, I investigate the effect of different environments (aquatic and terrestrial) on animal fitness, measured as growth rate (Michaels et al., 2014), and on the skin microbiome of captive alpine newts (*Icthyosaura alpestris*) reared in aquatic and terrestrial environments. I also use *in vitro* challenges between isolated bacteria and *Batrachochytrium dendrobatidis* (*Bd*) to predict effects of environment on susceptibility to infection by the fungus. This information may be used to guide *ex-* and *in-situ* management of amphibians and their diseases, as well as inform translocations between the two.

METHODS

Study animals and experimental conditions

F1 captive-bred *Ichthyosaura alpestris apuana* were obtained as eggs from a private breeder and were the progeny of a co-housed group of three males and three females laid over a period of three days. All animals used in the study hatched within three days of one another and larvae were reared to metamorphosis in a growth chamber at air temperature of 17 °C (day) and 15 °C (night) under a 12:12 photoperiod. Larvae were reared in large, bare-bottom aquaria thickly planted with live, growing plants (*Egeria densa* and *Fontinalis antipyretica*), which were filtered with air-driven sponge filters. Hatchling larvae were initially fed on *Artemia salina nauplii* and then on *Daphnia* spp., bloodworms (*Chironomidae*) and whiteworms (*Enchytraeus albidus*) until metamorphosis.

After metamorphosis, animals were maintained in groups of three in plastic vivaria (Faunarium 'large', Exo Terra – 370 x 220 x 250 mm) and were allocated alternately to terrestrial (n = 30) or aquatic (n = 30) conditions. Newts were maintained in these environments for the duration of the 12 month study. Terraria (housing terrestrial animals) had a substrate made from 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. Upturned plastic plant saucers with an entrance hole cut in the rim were provided as hides and both substrate and hides were covered with a layer of live moss. Terraria were planted with *Tradescantia* sp. Substrate was not changed throughout the study as its biological capacity to break down waste, along with live plants, preserved hygienic conditions. Aquaria had a substrate of organic aquatic mulm derived from decomposed plant matter, similar to a natural pond, which was present from the start of the study, were filtered using an air-driven sponge filter and thickly planted with *E. densa* and *F. antipyretica*. 10% partial water changes were performed weekly to maintain water quality. Experimental animals were broadcast fed on whiteworms and chopped earthworms.

Individual animals were identified using photographs of the markings on the ventro-lateral surface (similar to Mettouris et al., 2016, but by eye rather than computer matching, which was feasible due to the smaller number of individuals to distinguish per tank, i.e. three animals). Two animals in aquatic conditions died during the course of the study. These animals were replaced with non-experimental individuals to maintain equal stocking density.

Morphometrics

Animals were photographed against a scale and SVL (snout-vent length) and CL (caudal length) were measured in millimetres using the freeware ImageJ (NIH; http://imagej.nih.gov/ij/) at metamorphosis and at subsequent three-month intervals up to twelve months. Proportional CL was calculated as the CL divided by the SVL of newts; this was collected in order to look for differences in the size of the tail, the primary swimming organ, between terrestrial and aquatic newts.

Bacterial communities

Bacterial communities were collected at month ten from nine newts per treatment group (n = 9, N = 18) with only one newt sampled per tank; all individuals were removed and a random one was selected for sampling. Sterile nitrile gloves were worn throughout handling and changed between newts to minimise cross-contamination. Newts were rinsed twice on their dorsal and ventral surfaces using sterile bottled water to remove any transient (i.e. non-symbiotic) bacteria from their skin (Lauer et al., 2007). Newts were then swabbed 20 - 25 times to collect cutaneous bacterial communities using sterile Eurotubo swabs (Deltalab, Rubi, Spain). The dorsal and ventral regions of the body were swabbed separately to maximize coverage and bacterial growth. Swabs were placed into 1.5 ml sterile screw-top tubes containing 1 ml of 0.8% w/v $NaCl_2$ solution to facilitate subsequent culturing methods. Care was taken to ensure newts were not harmed during this process, and individuals were monitored for two weeks post-swabbing for signs of distress or injury in response to the swabbing (skin lesions, inappetence or condition loss, excessive skin secretions, or other unusual behaviour), of which none was observed.

Tubes containing swabs were vortexed to dissociate bacteria from the swab. The swab was removed and serial dilutions were constructed up to 10-1 by pipetting 100 ul into 900 ul of 0.8 % w/v $NaCl_2$, and dilutions of 100 and 10-1 were plated out on R2A agar media (Lab M Ltd., United Kingdom) and incubated at 15-17 °C (the same temperature at which the newts were maintained). Pilot studies showed these dilutions gave plates with an intermediate amount of growth that was most suitable for assessing the bacterial community (R.Antwis, unpublished data). New morphologically distinct bacteria colonies ('morphotypes') were counted three and seven days after plating, after which negligible new colony growth was observed. Bacterial counts were summed for the two counts (days three and seven), multiplied by the dilution factor of 10 where necessary, and counts were then averaged across the two dilutions.

Representative colonies of each morphotype were streaked out on R2A agar until a pure culture was obtained. Bacterial species were identified using 16S rDNA sequencing with universal primers 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACT-3') (Webster et al., 2003). 16s RNA fragments were obtained through colony PCR amplification using the Platinum PCR SuperMix (Invitrogen, Life Technologies) according to the manufacturer's instructions. DNA fragments were amplified by PCR using the following cycling parameters: 95 °C for 2

minutes followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, with a final extension step of 5 minutes at 72 °C. Prior to purification and sequencing, PCR products were checked for the correct length with gel electrophoresis. PCR products were purified with the GenElute™ PCR Clean-up Kit (Sigma-Aldrich®), and sequenced at the DNA Sequencing Facility, University of Manchester, UK. A consensus sequence was obtained by combining the forward and reverse sequences in DNA Dynamo Sequence Analysis Software© (BlueTractorSoftware Ltd., UK). Consensus sequences were then blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify each morphotype to genus level.

***In vitro Bd* challenges**

Pure cultures of each bacterial morphotype were used to conduct *in vitro Bd* challenges similar to Harris et al. (2006). The *Bd* strain GPL SFBC 014 (isolated from *Epidalea* (formerly *Bufo*) *calamita*, UK by Peter Minting in 2010) was grown in TGhL liquid media at 18 °C until maximum zoospore production was observed. Approximately 3 ml of the *Bd* zoospore culture was then spread across 1 % tryptone, 1 % agar plates and dried in a sterile hood. Bacterial cultures were streaked on either half of the plate (two per plate), inverted and incubated at 18 °C. Bacterial streaks were then scored for the presence or absence of a zone of inhibition, indicated by a markedly reduced or absent growth of *Bd*.

Statistical analyses

Aquatic and terrestrial newt SVL and proportional CL at day 0 (i.e. immediately prior to the start of the experiment) was compared using two-sample two-tailed T-tests. Newt SVL and proportional CL data were analysed using linear mixed-effect models in the lme4 and lmertest packages (Bates et al., 2015) in R using RStudio (R Core Team, 2021). Newt individual, nested within Tank, was used as a random factor to control for the repeated measures and nested elements of the design. Time and environment were used as explanatory variables. Prior to analysis, model assumptions were confirmed by visually inspecting residuals using the ggResidpanel function in R (Goode & Rey, 2019).

Differences in the overall bacterial community composition according to environment (terrestrial vs. aquatic), surface (dorsum vs. ventrum) and their interaction were analysed using the Adonis function of the vegan package in R using RStudio© (Oksanen et al., 2020). This function was used to compute a permutational multivariate ANOVA on the overall bacterial community structure (subsequently referred to as the Adonis analysis), using a Bray-Curtis distance matrix derived from the abundance of each morphotype (Antwis et al., 2014a; b; Michaels et al., 2014). As surface had no effect on the bacterial community (see Results), the data for the dorsum and ventrum of each newt were combined for subsequent analyses. The effect of environment on species richness (the number of different morphotypes on each individual) and total abundance (total number of cultured bacteria for each individual) were analysed using t tests in JMP 10®. Differences in the abundance of two bacterial strains that inhibited the *Bd* in *in vitro* challenge assays were analysed using t-tests in JMP 10®.

RESULTS

Effects of rearing condition on morphometrics

Neither SVL (t_{57}=0.40, p=0.69), nor proportional CL (t_{57}=1.5, p=0.14) differed between treatments at the start of the study. There was a significant effect of environment, such that aquatic newts grew larger than terrestrial animals ($F_{1, 17.9}$ = 15.7, p = 0.001; Fig. 1). SVL increased [mean(SD)] by 48(15) % and 35(12) % in aquatic and terrestrial newts, respectively, over the course of the study. Aquatic newts also had significantly proportionately longer tails than terrestrial newts ($F_{1, 18.3}$ = 128.54, p<0.001); mean (standard deviation) CL:TTL ratio at month 12 was 0.47(0.02) and 0.44(0.02) in aquatic and terrestrial newts, respectively (Fig. 1).

Effects of environment on cutaneous bacterial communities

A total of 11 different morphotypes (GenBank accession numbers KC853151-KC853154 and KF444805- KF444808; Table 1) were cultured from newts, with a range of 3 to 9 morphotypes per individual. All morphotypes were isolated from animals in both the terrestrial and aquatic environments, although the proportion of individuals hosting each morphotype was generally lower for newts in the aquatic set-ups (Table 1). Three bacteria could not be identified due to poor sequence data.

The results of the Adonis analysis showed there was a highly significant difference in the overall bacterial community associated with the skin of newts maintained in a terrestrial environment compared to those kept in an aquatic environment ($F_{3, 32}$ = 25.695, p = 0.001), but no significant effect of surface (dorsum or ventrum; $F_{3, 32}$ = 0.827, p = 0.480) or the interaction between surface and environment ($F_{3, 32}$ = 0.744, p = 0.509) were detected. Newts kept in a terrestrial environment supported a significantly greater bacterial abundance (t_{17} = 6.119, p < 0.001; Fig. 2) and significantly greater species richness (t_{17} = 3.714, p < 0.001; Fig. 3).

Two of the 11 morphotypes successfully inhibited *Bd* in the *in vitro* challenges; *Arthrobacter* sp. (KC853151) and *Chryseobacterium* sp. (KF444806). Both of these were isolated from all or nearly all individuals in both environmental groups (see Table 1), although the total abundance of each bacterium was significantly higher on the skin of newts maintained in a terrestrial environment (*Arthrobacter* sp., $t_{1,8}$ = 3.83, p = 0.003; *Chryseobacterium* sp., $t_{1,8}$ = 2.13, p = 0.030; Fig. 4).

DISCUSSION

Here I show that newts reared in an aquatic environment support a simpler and less abundant bacterial community than their terrestrial counterparts. Although bacterial communities are acquired through interactions with the environment (Belden & Harris, 2007; Banning et al., 2008; Walke et al., 2011; Daskin & Alford, 2012), the rinsing

Table 1. Bacteria morphotypes isolated from skin swabs of 18 alpine newts (*Ichthyosaura alpestris apuana*) and their potential anti-*Bd* activity. Proportion of aquatic and terrestrial newts carrying each type of bacterium is provided.

Species	Anti-Bd activity	Proportion terrestrial newts (n=9)	Proportion aquatic newts (n=9)
Arthrobacter sp. (Ia1)	Yes	1.0	0.89
Unidentified (Ia2)	No	1.0	1.0
Erwinia sp. (Ia3)	No	0.78	0.14
Chryseobacterium sp. (Ia4)	Yes	1.0	1.0
Flavobacterium sp. (Ia5)	No	0.56	0.14
Lysobacter sp. (Ia6)	No	0.78	0.14
Unidentified (Ia7)	No	0.89	0.14
Rhizobium sp. (Ia8)	No	0.66	0.28
Flavobacterium sp. (Ia9)	No	0.44	0.33
Unidentified (Ia10)	No	0.28	0.89
Shewanella sp. (Ia16)	No	0.28	0.14

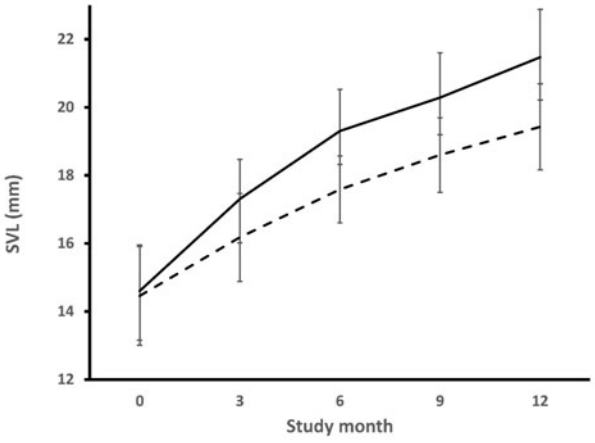

Figure 1. Growth of aquatic (solid line) and terrestrial (dashed line) alpine newts over the duration of the study. Error bars represent +/- 1SEM.

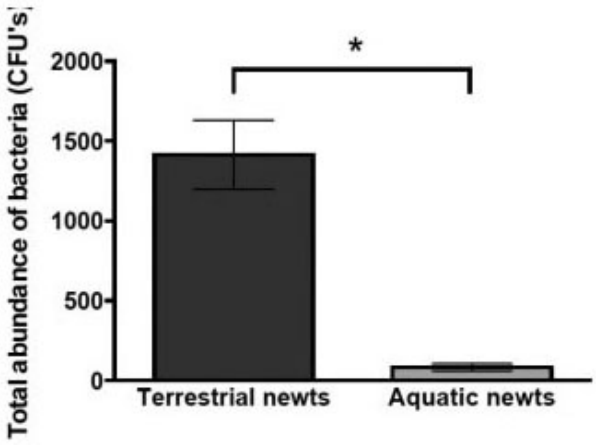

Figure 2. Total abundance of bacteria isolated from skin swabs of 9 terrestrial and 9 aquatic newts. Error bars represent +/- 1SEM.

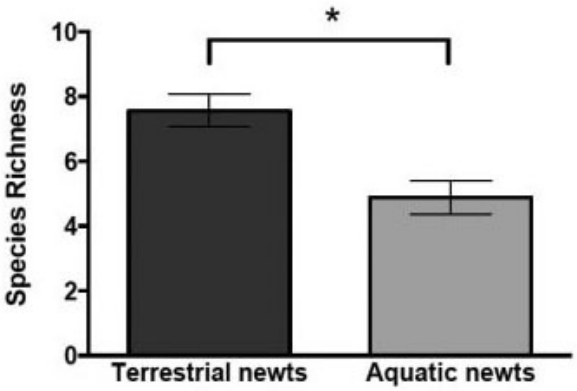

Figure 3. Mean species richness of skin bacterial communities isolated from skin swabs of 9 terrestrial and 9 aquatic newts. Error bars represent +/-1SEM.

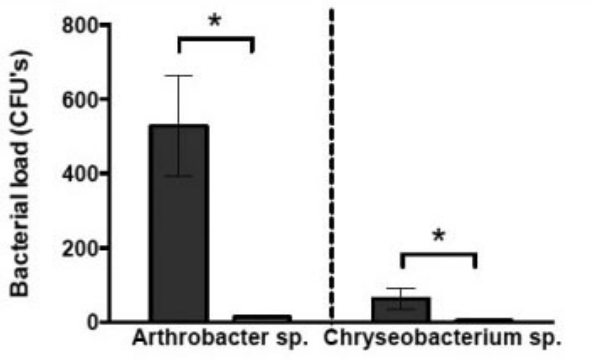

Figure 4. Prevalence of anti-Bd bacteria (*Arthrobacter* sp. and *Chryseobacterium* sp.) isolated from skin swabs of 9 terrestrial (left-hand column in each pair) and aquatic (right-hand column) newts. Error bars represent +/-1SEM.

part of the swabbing protocol is designed to remove transient environmental bacteria (Lauer et al., 2007). Simple differences in the bacterial abundance of aquatic and terrestrial environments, if these existed (we did not measure this), are therefore unlikely to have directly accounted for the difference in cutaneous communities detected, unless complex interactions inhibited or promoted the growth of certain bacteria. Therefore, the difference found in the skin microbiome between newts from aquatic and terrestrial environments may reflect the more textured and less frequently sloughed skin of terrestrial newts (pers. obs.), which may offer more niches and less disturbance for bacterial colonisation, as well as abiotic impacts of the differing environments on bacterial survival and growth.

More diverse and numerous cutaneous bacterial communities may be more robust and offer more protection against pathogens (Matos et al., 2005; Belden & Harris, 2007; van Elsas et al., 2012; Eisenhauer et al., 2013). I report in this study that the dermal bacterial communities harboured by newts included two species that inhibited the growth of *Bd in vitro*; *Arthrobacter* sp. and *Chryseobacterium* sp., and aquatically reared newts hosted lower abundances of these *Bd*-inhibiting bacteria than terrestrially reared newts. Higher density of bacteria on terrestrial newts may facilitate anti-pathogenic activity through quorum sensing, or due to minimum inhibitory concentrations of peptides required to suppress pathogen activity (Miller & Bassler, 2001; Brucker et al., 2008a; b).

The dermal bacterial communities harboured by newts included two species that inhibited the growth of *Bd in vitro*; *Arthrobacter* sp. and *Chryseobacterium* sp., and aquatically reared newts hosted lower abundances of these *Bd*-inhibiting bacteria than terrestrially reared newts. *Bd* is thought to be carried between ponds by *I.alpestris*, particularly where it has been introduced in the UK (Fisher & Garner, 2007; Duffus & Cunningham, 2010) where the strain of *Bd* used in this study originated. The presence of *Bd*-inhibiting bacteria on both phases of the newts (albeit in varying degrees) may help to explain the ability of this species to carry *Bd* infection without succumbing to disease. If aquatic phase alpine newts have less complex bacterial communities with a reduced capacity to inhibit *Bd*, this may increase the potential for alpine newts to communicate disease to other species present at breeding sites, as well as rendering them more susceptible to infection by *Bd*. However, given the effects of captivity on natural bacterial communities in amphibians in comparison to wild populations (Loudon et al. 2013; Antwis et al., 2014; Becker et al., 2014; Michaels & Preziosi, 2020), it is unclear whether wild animals will show similar differences in the bacterial communities associated with aquatic and terrestrial individuals.

In other amphibians, the number of individuals infected with *Bd* increases during breeding assemblages in water bodies (Kriger & Hero, 2007; Voordouw et al., 2010; Minting, 2012), as does the infection load of individual animals (Retallick et al., 2004). My data may suggest an additional mechanism for this (reduction in *Bd*-resistant microbiome under aquatic conditions), but this inference cannot be robustly made as I did not study animals transitioning between environments, either following metamorphosis or returning to the water to breed. Moreover, my data does not address the speed at which bacterial communities adjust in response to the change between aquatic and terrestrial habitats and this may be important in inferring epidemiological implications. In addition, although it is known that moving amphibians from the field to captivity influences microbiome (e.g. Becker at al., 2014; Bates et al., 2019), further work is required to investigate the impact of translocation into the wild on cutaneous bacterial communities of amphibians, and whether patterns established in captivity are maintained in the wild. My data correlates with those presented by Daversa et al. (2018), who showed that routine switching of alpine newts between terrestrial and aquatic habitats across their reproductive cycle reduced *Bd* growth, and heavily infected animals spent more time on land.

I also found that aquatic juveniles grew larger than terrestrial efts over the course of the twelve month experiment. This is similar to field observations of another newt species, *Notophthalmus viridescens* (Healy, 1973) and may reflect higher metabolic rates in the aquatic phase (Kristín & Gvoždík, 2013), coupled with reduced energetic cost of aquatic locomotion (Shaffer et al., 1991). This hypothesis is supported by the proportionately longer tails of aquatic newts, which is likely also an adaptation to aquatic locomotion, as seen in adults other newts (Treer & Treer, 1995). More rapid juvenile growth is linked to increased adult size in a number of amphibians (Jørgensen, 1986; Semlitsch et al., 1988; Altwegg & Reyer, 2003), which has been shown to have important impacts on survivorship (Clarke, 1974; Kusano, 1981; Bardsley & Beebee, 1998; Altwegg & Reyer, 2003) and reproductive success (Jørgensen, 1986; Briggs, 2013; Yeager & Gibbons, 2013). There is therefore strong potential for lifelong effects of juvenile environment on body size and therefore on fitness. Captive populations of newts are often reared aquatically as efts, where possible, due to the ease of feeding (aquatic newts will take defrosted frozen foods and pellets; pers. obs.; Pasmans et al. (2014)) and of cleaning aquaria. It is therefore important to understand the impacts of this practice on the fitness of populations that are candidates for translocation. Rearing newts aquatically may therefore improve both the efficiency of breeding programmes and the potential for reintroduction success in terms of survivorship and reproductive output. However, according to my data, this may come at the cost of reduced disease resistance through a reduction in cutaneous bacterial community diversity and abundance. This trade-off is in contrast with work in anurans (Ogilvy et al., 2012; Antwis et al., 2014, Michaels et al., 2014), which has found associations between factors of captive husbandry that improve growth rates as well as the diversity of cutaneous bacterial communities. This highlights the importance of using multiple biological measures to assess the effects of different captive environments on fitness traits of amphibians.

In this study I have shown the environment in which juvenile newts develop has significant effects on growth rates and cutaneous bacterial communities. Although bacterial culturing methods are known to underestimate

species richness and bacterial abundance (reviewed in Amann et al., 1995), genetic methods were outside of the feasible scope of this study. Nevertheless, these data provide an insight into the effects of environment on dermal bacterial communities that are likely to transcend to the non-culturable parts of the community. These differences may influence survivorship, adult size and reproductive fitness, as well as the part played by *I. alpestris* in disease spread, and as such have important implications for both *ex-* and *in-situ* conservation.

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Ethical statement

All methods used in this study were non-invasive and did not require a UK Home Office Licence. The University of Manchester Ethics Committee approved this study prior to commencement.

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