Population effects on the epidermal bacterial colonies of *Alytes muletensis*

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Introduction

Amphibians across the globe are experiencing declines due to habitat loss, climate change, anthropogenic influences, and disease (Stuart et al. 2004). One of the major threats to amphibians is the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd)* (Berger et al. 1998; James et al. 2009; Fisher et al. 2012) the causative agent of amphibian chytridiomycosis disease (Berger et al. 1998). This pathogen has been detected on the island of Mallorca where the Mallorcan midwife toad, *Alytes muletensis,* is still recovering from previous population loss (Serra et al, 2009). Given the current lack of a wide-reaching treatment for chytridiomycosis in the wild, *ex situ* populations of vulnerable species are being established in order to alleviate the risk of extinction under the Amphibian Ark initiative (Gascon et al. 2005). In the meantime, research is focusing on the potential for symbiotic bacteria to act as a probiotic treatment against *Bd* in the wild (eg. Becker et al. 2011; Bletz et al. 2013; Harris, Brucker, et al. 2009; Woodhams et al. 2011). Amphibian skin plays host to a plethora of microorganisms (Culp *et al.*, 2007; Becker *et al.*, 2011; Bletz *et al.*, 2013; Roth *et al.*, 2013; Antwis *et al.*, 2014; Michaels *et al.*, 2014) and symbiotic bacteria on the epidermis are the only known non-host produced line of defense against pathogens (Woodhams *et al.* 2007; Becker *et al.*, 2011; Bletz *et al.*, 2013). Recent studies have shown that some symbiotic bacteria, such as *Janthinobacterium lividum*, have anti-*Bd* properties, with subsequent potential to influence disease outcome of hosts (reviewed in Bletz *et al.*, 2013). Manipulation of host microbiome may prove to be a practical solution to mitigating *Bd* in the wild.

Research has shown that populations of amphibians have varying rates of mortality and infection from pathogens such as *Bd* (Fisher et al. 2009). For example, some populations of *Rana muscosa* in North America can persist despite infection from *Bd,* whilst other populations have seen dramatic declines with some local extinctions occurring (Briggs et al. 2014). There have been several suggested reasons for this disparity including differences in the abiotic influences on the populations and varying abilities to host an immune response (Briggs et al. 2014). One study has shown that in captivity varying light levels influence the richness and abundance of epidermal bacteria and cutaneous peptide profile by affecting the faecal glucocorticoid metabolite concentration (Jogee et al., unpublished data). Circulating glucocorticoid can block the production of cutaneous antimicrobial peptides, which in turn alters the bacteria present on the skin. In the wild, however, individuals living in close proximity and therefore experiencing similar abiotic factors have shown varying abilities to resist infection. This may imply there may be more than abiotic factors affecting epidermal microbiota and thus disease resistance.

First proposed by Antonovic the field of community genetics aims to unify the fields of ecology and evolutionary biology by looking at how the genetic variation and evolution of one species can shape whole communities (Neuhauser et al. 2003). By understanding the relationship between ecology and evolutionary biology we can begin to understand the driving factors of the abundance and phenotypic diversity within and amongst species (Hersch-Green et al. 2011). For example, by using experimental crosses of poplar trees *Populus angustifolia* and *Populus fremontii*, which produce varying levels of condensed tannins (thought to act as an anti-herbivory mechanism) (Kraus et al. 2003) research has shown that poplars are a foundation species and their phenotypes drives community structure and ecosystem processes (Whitham et al. 2006). Firstly, the concentration of tannin affects the arthropod community associated with the tree (Shuster et al. 2006), which in turn influences the avian predators that visit the tree (Whitham et al. 2006). Secondly, the condensed tannins in the leaf litter are associated with a reduction in decomposition, nutrient release and nitrogen mineralization (Schweitzer et al. 2014). The disruption of nitrogen cycling can feedback on the poplar, which may need to invest more in fine-root production to increase nutrient uptake (Whitham et al. 2006). So far most studies into community genetics have used plant foundation hosts due to their ease of manipulation, but it is unlikely that this phenomenon is restricted to plants. This study aims to test whether there is population effect on the bacterial strains that colonise amphibian skin. If there is then this could be partly why some populations of amphibians succumb to infection more readily than others.

Alytes muletensis will be used as a model species in this study for several reasons. Firstly, as the males carry egg clutches from individual females so clutch effects can be reliably looked at (occasionally they will carry more than one clutch, but this can be determined as the eggs of one female are attached together by a mucoidal string). Secondly, ZSL London Zoo Living Collections have kept a detailed record of the lineage of their *A. muletensis* population and individuals collected from 3 different populations have been maintained separately since collection. Thirdly, *A. muletensis* tadpoles are large enough to swab their mouthparts, the site of *Bd* infection in tadpoles (Densmore & Green 2007). Finally, *A. muletensis* is a Red List vulnerable species with *Bd* being a threat in its natural range of Mallorca (Serra et al. 2009). Information gleaned from this project will contribute both to the captive management of the species and also contribute to the growing knowledge of intraspecies variation in disease resistance. Adding to our understanding of amphibian epidermal bacterial communities is vital if we are to use probiotics to mitigate diseases in the wild both here in the UK and overseas.

Method

Four clutches (A-D) of *A. muletensis,* representing 3 populations (A- C) were bred over the summer breeding period 2014 in the living collection at ZSL London Zoo. The three populations were originally collected from Mallorca from three different torrents along one stream. Each clutch was maintained in a basic tank, with filter and pipe off-cut to provide shelter. Tadpoles were fed daily with flaked fish food and weekly 10% water changes performed. On the 2/10/14, tadpoles had reached a large enough size to be VIE tagged. One swab was taken from each of the tadpole tanks to sample bacteria present in the water. In order to ensure that there was adequate replication both between and within tanks twelve tadpoles were randomly chosen from clutch A, twelve from clutch B, six from clutch C and six from clutch D. These tadpoles were individually removed from their tanks, rinsed using sterilised water and swabbed 10 times on both their ventral skin surface and their mouthparts using separate charcoal media transwabs. Six experimental tanks were set up similar to the previous growth tanks, but now the water was homogenised across the tanks by removing 3 litres of water from each tank, mixing it in a bucket and then redistributing the water equally across the 6 tanks. To amplify the amount of bacteria in the water 750 ml of water mixed with detritus from the parental tanks was added to the tanks. Before adding this to the tanks a swab was taken from the detritus water to sample the bacteria present in the water and to allow comparison between the detritus and the growth tank water. (This addition of detritus was then replicated once weekly after the 10% water change to maintain the increase in bacteria.) Tadpoles were then VIE tagged so they could be later identified. The two groups of twelve tadpoles were split in half so that there was six different groups of six tadpoles which were all tagged a separate colour (white, blue, orange, pink, red and yellow). Tadpoles were then arranged so that there was one individual of each colour in the 6 experimental tanks. Four weeks later on the 30/10/14 tadpoles were swabbed again as previously described.

All swabs from this study were plated directly onto R2A agar media and incubated for 48 hrs at 24˚C to allow colony forming units (CFUs) to develop. Different CFUs morphotypes were identified and were counted and recorded for each plate. Bacterial abundance and richness were calculated in an Excel spreadsheet and statistical analysis was performed in R.

Results

Population Effect on Bacterial Colonies

On the 2/10/14 seven culturable bacterial CFU morphotypes were isolated from tadpole skin and mouthparts. On the 30/10/14 the same seven were isolated from the tadpoles plus two previously undetected CFU morphotypes (see Table's 1 and 2). Five tadpoles died after the VIE tagging procedure.

2/10/14		Morphotype						
Tissue	Population	1	2	3	4	5	6	7
Skin	A	X	X	X	X	X	X	
	B	X	X	X	X	X	X	
	C	X	X	X	X	X	X	
Mouthparts	A	X	X	X	X		X	X
	B	X	X	X		X		
	C	X	X	X	X	X	X	

Table 1) Showing the community structure of the different tissue types for the three populations sampled on the 2/10/14. Morphotypes 1,2 and 3 were common species being isolated from all populations and all tissue types. Morphotype 7 was the rarest species only being found on the mouthparts of population A.

30/10/14		Morphotype									
Tissue	Population	1	2	3	4	5	6	7	8*	9	10
Skin	A	X	X	X		X	X	X			
	B	X		X	X		X	X		X	X
	C	X	X	X		X	X	X			
Mouthparts	A	X	X	X	X	X	X	X			
	B	X	X	X	X		X	X		X	
	C	X	X	X	X	X		X			

Table 2) Showing the community structure of the different tissue types for the three populations sampled on 30/10/14. * Morphotype 8 was not isolated from the tadpoles, however it was detected in both Tank 1 (23 CFU's) and Tank 2 (11 CFU's). Morphotype 2 was no longer present on the skin of population B, perhaps due to competition from the introduced bacterial species. Although previously a rare species, morphotypes 7 is now detected on all tissue types in all populations.

Using a generalised linear model with Poisson distribution blocking for Tank effects in R, on the 2/10/14 population had a significant effect on the abundance of bacterial colonies of the mouthparts (n = 35) and skin of tadpoles (n = 35) (X^2 = 1273.8, df = 2, p = <0.001 and X^2 = 214.16, df = 2, p= <0.001 respectively, see Fig. 1). Population had a significant effect on the richness of bacterial colonies of the mouthparts, but not the skin of tadpoles (X^2 = 9.08, df = 2, p = 0.01 and X^2 = 0.67, df = 2, p = 0.71, respectively, see Fig. 1).

Using a generalised linear model with Poisson distribution blocking for Tank effects in R, on the 30/10/14 population had a significant effect on the abundance of bacterial colonies of mouthparts (n = 23) and skin of tadpoles (n = 16) (X^2 = 228.11, df = 2, p = <0.001 and X^2 = 129.71, df = 2, p = <0.001 respectively, see Fig. 1). However, population did not have a significant effect on the bacterial richness of either the mouthparts or skin of tadpoles (X^2 = 3.11, df = 2, p = 0.21 and X^2 = 0.05, df = 2, p = 0.98 respectively, see Fig. 1).

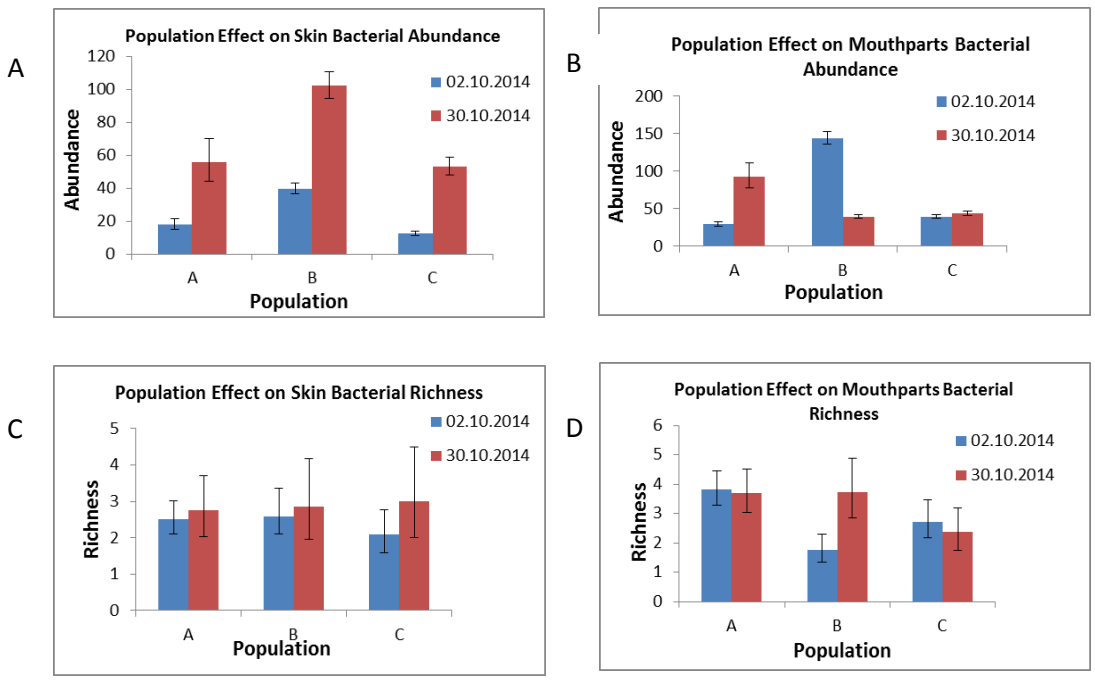


Fig. 1 A) Population B has the highest bacterial abundance of the skin on both dates of the study; it also has the largest increase in abundance going from a mean of 39.65 to 102.51 per tadpole although all populations see an increase. B) However, the bacterial abundance of the mouthparts of population B decreases from the 2/10/14 to the 30/10/14 whilst population A increases and population C stays relatively stable. C) The richness of the skin stayed relatively stable although composition of the communities will have changed (see Table 1 and 2)

Differences between Mouthparts and Skin

Using the t-test function in R, on the 2/10/14 the mouthparts (mean = 74.31 ±12.70) had significantly higher abundance of bacterial colonies than the skin of tadpoles (mean = 25.09 ±4.39) (t = -3.7683, df = 43.254, p = <0.001). There was no significant difference in the richness of bacterial colonies between mouthparts (mean = 2.74 ±0.23) and skin of tadpoles (mean = 2.4 ±0.16) (t = 1.28, df = 34, p = 0.21, see Fig. 2).

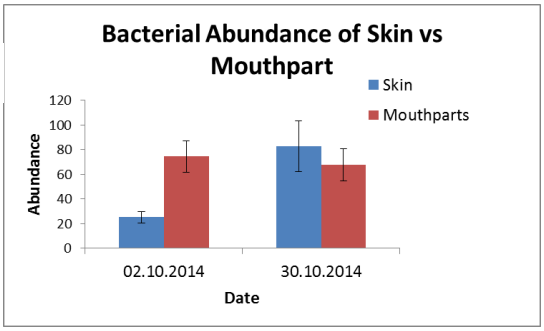
Using the t-test function in R, on the 30/10/14 there was no significant difference in the bacterial abundance of tadpole mouthparts (n = 23, mean = 67.74 ±13.12) compared to tadpole skin (n= 16, mean = 82.5 ±20.55) (t = -1.56, df = 15, p = 0.14, see Fig. 2). There was also no significant difference between bacterial richness of the mouthparts (mean = 3.68 ±0.28) and the skin (mean = 2.88 ±0.26) of tadpoles (t = 0.88, df = 15, p = 0.39, see Fig. 2).

Effect of Treatment on Abundance and Richness

Using the paired t-test function in R there was no significant difference in the abundance of bacterial colonies on the mouthparts on the 2/10/14 (mean = 74.31 ±12.70) compared to 30/10/14 (mean = 67.74 ±13.12) (t = 0.2915, df = 23, p = 0.7733, see Fig 2.). There was a significant difference in the abundance of bacterial colonies on the skin on the 2/10/14 (mean = 25.09 ±4.39) compared to the 30/10/14 (mean = 82.5 ±20.55) (t = -2.4408, df = 16, p = 0.02666, see Fig 2.).

Using the paired t-test function in R there was no significant difference in the richness of bacterial colonies of the mouthparts of tadpoles on the 2/10/14 (mean = 2.74 ±0.23) compared to the 30/10/14 (mean = 3.68 ±0.28) (t = -1.8427, df = 23, p = 0.0783, see Fig 2). There was no significant difference in the richness of bacterial colonies of the skin on the 2/10/14 (mean = 2.4 ±0.16) compared to the 30/10/14 (mean = 2.88 ±0.26) (t = -1.4822, df = 16, p = 0.1577, see Fig. 2).

A



B

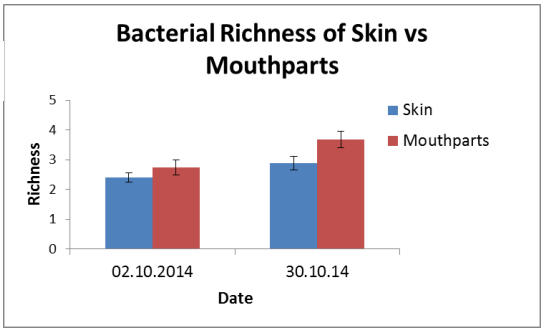


Fig 2. Comparing the means of bacterial abundance (A) and bacterial richness (B) for skin and mouthparts and also showing the changes over time. Bacterial abundance of mouthparts stayed relatively stable across the study (A), but for the skin saw a large increase in CFU's. Bacterial richness stayed stable for the skin of tadpoles across the study, whilst for mouthparts it significantly increased (B).

Discussion

This study has shown that population has an effect on the abundance of bacterial CFU's of both skin and mouthparts. Richness was significantly affected by population at the beginning of the study; however this may be due to an environmental effect as the influence on richness was not maintained throughout the study. Until the start of the study the clutches had all been kept separate meaning

that the different tanks may have had varying bacterial composition, which would have affected richness. After the treatment richness across the study became very similar, this is likely due to the homogenising and redistributing of tank water. It is therefore unlikely population effects richness in the same way that it affects abundance. What it does however shed light on is that despite appearance the tanks were in fact different having implications for husbandry. Efforts should be made to make sure that water is homogenised across tadpole tanks to ensure a varied microbiota is introduced to all individuals. This is particularly important if tadpoles are to be released into the wild in area where *Bd* has been detected.

The fact that population has an effect on bacterial abundance suggests that certain populations of tadpoles provide an environment that encourages bacterial growth. What we are describing here as a population affect may also be attributed to a maternal or even paternal effect. Bacteria are passed from female to egg during the laying of clutches, a female with a more diverse and abundant microbiota will pass this onto her eggs. However, males of this species carry the eggs until they hatch so there may also be vertical transmission of bacteria from the male to the eggs. Mating pairs that both have a more complex microbiota in captivity may be produce tadpoles with a similar microbiota. This may be important in the captive management of *A. muletensis* in the future. The Amphibian Ark initiative aims to eventually release captive bred individuals back into the wild, those that are better at resisting infection will have a better chance of survival. As amphibians are often release as tadpoles in a process known as 'head-starting' (Griffiths & Pavajeau 2008) it is therefore important to understand what influences the tadpole microbiome particularly if it is an influence from when the eggs are first laid.

Population B is interesting in that the mouthparts saw a significant decrease in bacterial abundance whilst the skin did not. On the first day of data collection population B had a significantly higher bacterial abundance than population C, but by the second day of data collection population B's abundance was more in line with that of population C. Population B was the only one to have morphotype 9 present, however when detected on the skin it was also in the presence of morphotypes 10 and not morphotype 2, whereas the reverse can be said for when it was detected on the mouthparts. There could possibly be an interaction occurring between these three species of bacteria meaning that when morphotype 9 and 10 are together their abundances increase, but when 9 is in the presence of morphotypes 2 their abundances decrease due to higher competition between these two species. Understanding how different bacterial strains interact is extremely important if using probiotics to mitigate disease is to be successful. A more abundant and diverse bacterial community has shown to be more stable (Matos et al. 2005) and therefore more adept at fighting infection (van Elsas et al. 2012), a reduction in abundance could jeopardise this ability.

On day one of the study mouthparts had significantly higher bacterial abundance than skin although this was no longer the case by day two of data collection. The increase in bacterial abundance of the skin is likely to be a reflection of the increase in bacterial abundance of the water. The bacterial abundance of the mouthparts stayed stable throughout the study, possibly due to more regulation of the bacteria growth on the mouthparts than on the skin. Understanding what regulates this stability could also be important to choosing tadpoles for head-starting release programmes. Although *Bd* is not lethal in tadpoles it can spread throughout the body at metamorphosis at which point it can quickly cause death (Densmore & Green 2007). Those with a more robust oral microbiota may be better at resisting Bd in the wild.

An interesting observation on the second round of sampling was that there was a large amount of swarmed colony plates, likely due to *Proteus mirabilis sp.* This reduced the sample sizes for the 30/10/14. This is a common bacillus species and is found in most soil and water bodies. It has the effect of swarming the entire plate making it impossible to count individual CFU's and even harder to sample for pure streaks to do PCR analysis.

Overall this study has become as excellent springboard for future study. The work done here is only what we know from the culturable bacteria. It is likely that there are countless more bacteria strains living on the skin of tadpoles and metagenomics analysis would be able to reveal these and we would be able to further understand the complexity of the tadpole microbiome. PCR and 16-s ribotyping of the culturable bacteria may also add towards establishing a core microbiome for these animals. This is the idea that there is a set of bacteria that are common and vital to the healthy functioning of amphibian skin. Some of these bacteria both culturable and non-culturable may be effective at killing *Bd.* To assess this first *in vitro* tests should be done in the lab to show which strains are effective, then work may be done to understand how we can transfer these safely and with longevity to the animal model in order to manipulate amphibian microbiome to mitigate *Bd.* It will also be important to understand the interaction between bacterial species. In this present study there may be a three-way interaction present between morphotypes 2, 9 and 10 influencing their abundance, but further *in vitro* tests would need to be done to show whether or not this is true. Finally and most interestingly, further research should be done to understand the population effect on bacterial abundance. Is it a maternal effect or is there a genetic underpinning to the differences in abundance. Could for example different populations have different epidermal antimicrobial skin peptides affecting the bacterial abundances? If so then tadpoles may prove to be a useful animal model for studying community genetics furthering our understanding of ecology, evolution and coevolution.

Acknowledgements and Achievements

I would like to formally thank PTES for awarding me the funding to undertake this project. This internship has given me a solid grounding for postgraduate study. I have designed a PhD project based around my work on this internship and I am currently looking for funding with several possibilities which may prove fruitful. The project will be looking at controlling infectious disease through the manipulation of host microbiomes and will look towards answering some of the questions brought up by my internship project such as, 'what is the source of the population effect on amphibian epidermal bacterial communities?'.

I would also like to thank the Institute of Zoology's Evolution and Molecular Ecology team and The Zoology Society of London Zoo's Reptile House Team, both for their patience and guidance on this project. Without their expertise I would not have been able to complete this project. I have learnt a great deal during my four months with them and look forward to working with them again. I hope to return in the spring when the tadpoles will be metamorphosing and I can look to see if the same bacterial species are carried through to the juvenile stage.

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