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The influence of dietary carotenoid supplementation on the cutaneous bacterial communities of the critically endangered Southern Corroboree Frog (Pseudophryne corroboree)

Casey Edwards

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The influence of dietary carotenoid supplementation on the cutaneous bacterial communities of the critically endangered Southern Corroboree Frog (Pseudophryne corroboree)

Abstract

Amphibians support a diverse range of cutaneous bacteria that may contribute to innate immune defence through mutualistic associations. These associations, which have come about through the coevolution of bacteria and amphibians, are potentially capable of supressing infection from deadly skin pathogens such as *Batrachochytrium dendrobatidis*. Conditions experienced within *ex-situ* conservation programs may affect the composition of the cutaneous bacterial community. In particular, nutritional conditions such as the provision of dietary carotenoids may influence cutaneous bacterial communities and have immune response implications for amphibians in captivity and post-release. Carotenoids exhibit efficient biological antioxidant activity and are known to influence vertebrate immune function through enhancing proliferation and functioning of immune response components. Vertebrates are unable to biosynthesize carotenoids *de novo* and must acquire these compounds via dietary means. This study aims to: 1) characterise the cutaneous bacterial community of a captive colony of the critically endangered Southern Corroboree Frog (*Pseudophryne corroboree*), and 2) test the effect of dietary carotenoid supplementation on these cutaneous bacterial populations. Dietary carotenoid availability was manipulated throughout juvenile (tadpole) and post-metamorphic (frog) life stages. Bacterial culturing methods were applied to frog skin swabs and bacteria were isolated and identified to determine the effect of dietary carotenoid supplementation on cutaneous bacterial communities. As expected, the provision of dietary carotenoids significantly increased bacterial abundance and species richness, and also affected overall community composition of bacteria. These findings provide support for the hypothesis that dietary carotenoid supplementation can enhance the cutaneous bacteria community of amphibians. It is expected that carotenoid supplementation enhances the cutaneous bacterial community by: 1) bacteria utilising carotenoids directly for physiological functions and/or; 2) the provision of a more suitable microhabitat for bacteria to reside through host utilisation of dietary carotenoids. Outcomes of this study contribute to a body of empirical evidence demonstrating the benefits of developing standardised *ex-situ* breeding conditions to maximise the mutualistic properties of cutaneous bacterial communities. This knowledge has the potential to improve the immune capabilities of amphibians both within captivity and upon release, and potentially aid in the suppression of amphibian pathogens.

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The influence of dietary carotenoid supplementation on the cutaneous bacterial communities of the critically endangered Southern Corroboree Frog (*Pseudophryne corroboree*)

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the Bachelor of Environmental Science Advanced (Honours)

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Faculty of Science, Medicine and Health
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Declaration

The information in this thesis is entirely the result of investigations conducted by the author, unless otherwise acknowledged, and has not been submitted in part, or otherwise, for any other degree or qualification.

Casey Edwards

29/09/2015

Thesis Abstract

Amphibians support a diverse range of cutaneous bacteria that may contribute to innate immune defence through mutualistic associations. These associations, which have come about through the coevolution of bacteria and amphibians, are potentially capable of supressing infection from deadly skin pathogens such as *Batrachochytrium dendrobatidis*. Conditions experienced within *ex-situ* conservation programs may affect the composition of the cutaneous bacterial community. In particular, nutritional conditions such as the provision of dietary carotenoids may influence cutaneous bacterial communities and have immune response implications for amphibians in captivity and post-release. Carotenoids exhibit efficient biological antioxidant activity and are known to influence vertebrate immune function through enhancing proliferation and functioning of immune response components. Vertebrates are unable to biosynthesize carotenoids *de novo* and must acquire these compounds via dietary means. This study aims to: 1) characterise the cutaneous bacterial community of a captive colony of the critically endangered Southern Corroboree Frog (*Pseudophryne corroboree*), and 2) test the effect of dietary carotenoid supplementation on these cutaneous bacterial populations. Dietary carotenoid availability was manipulated throughout juvenile (tadpole) and post-metamorphic (frog) life stages. Bacterial culturing methods were applied to frog skin swabs and bacteria were isolated and identified to determine the effect of dietary carotenoid supplementation on cutaneous bacterial communities. As expected, the provision of dietary carotenoids significantly increased bacterial abundance and species richness, and also affected overall community composition of bacteria. These findings provide support for the hypothesis that dietary carotenoid supplementation can enhance the cutaneous bacteria community of amphibians. It is expected that carotenoid supplementation enhances the cutaneous bacterial community by: 1) bacteria utilising carotenoids directly for physiological functions and/or; 2) the provision of a more suitable microhabitat for bacteria to reside through host utilisation of dietary carotenoids. Outcomes of this study contribute to a body of empirical evidence demonstrating the benefits of developing standardised *ex-situ* breeding conditions to maximise the mutualistic properties of cutaneous bacterial communities. This knowledge has the potential to improve the immune capabilities of amphibians both within captivity and upon release, and potentially aid in the suppression of amphibian pathogens.

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1. Introduction

1.1. Amphibian extinction crisis and the role of ex-situ conservation programs

Rates of amphibian species decline and extinction are unprecedented and far exceed all other vertebrate classes. Globally, more than 30% of amphibian species are facing extinction and several synergistic factors have been identified as significant causative agents (IUCN, 2015). These factors include disease, habitat loss and degradation, introduced species and climate change. In addition, a prominent threat to amphibian biodiversity is the rapid emergence of *Batrachochytrium dendrobatidis* (Bd), a pathogenic fungus which colonises the skin of amphibians and causes the lethal disease chytridiomycosis (Holden et al., 2015; Lam et al., 2010). Amphibians are particularly sensitive to environmental perturbations and pathogen infection, a vulnerability which predominantly results from the porous structure of amphibian skin (Bletz et al., 2013; Kueneman et al., 2014). However, this unique skin structure also enables the development of beneficial components of innate immunity, including mutualistic interactions with cutaneous bacteria (Kueneman et al., 2014; Lauer et al., 2007).

Amphibians are critical to ecosystem functioning and biodiversity, perform vital roles as indicator species and provide information that is of medical significance to humans (Rollins-Smith & Woodhams, 2011). These factors, along with a general ethical obligation to preserve biodiversity, underscore the necessity to help maintain amphibian species into the future. As a result of rapid species declines, *in-situ* conservation efforts alone are often insufficient in maintaining viable amphibian populations. Ameliorating threats *in-sit*u is a complex and time consuming process which may extend beyond the timeframe of an amphibian species decline to extinction (Gascon, 2007). As a result, the establishment and implementation of *ex-situ* conservation programs is often necessary to ensure the persistence of amphibian species, as highlighted by the Amphibian Conservation Action Plan (ACAP)(Gascon, 2007). *Ex-situ* programs allow for the enhancement of population numbers via the exclusion of key threatening processes which have caused the species decline. However, these programs can fail to re-establish healthy wild populations if the key threatening processes are not eliminated or managed (Gascon, 2007).

Ex-situ conservation programs often attempt to mimic conditions experienced in the wild so as to avoid the development of maladaptive traits, and prepare individuals for release back into the wild. However, these 'wild' conditions are not always well understood (Becker et al., 2014). Husbandry conditions experienced in captivity (e.g. diet) are likely to impact the immune response of frogs within the captive population (Antwis et al., 2014; Becker et al., 2014; Meyer et al., 2012; Michaels et al., 2014). However, the influence of various captive conditions on amphibian immune function is not well understood and thus warrants further investigation. Amphibian conservation research must target the development of standardised captive husbandry conditions which maximise the immune function and health of amphibians both in captivity and upon release. These efforts will help in the suppression of deadly pathogen infections, perhaps even *B. dendrobatidis*, and assist in re-establishing healthy wild populations (Becker et al., 2014). Amphibian skin is a major area of interest as it is the first line of defence against most pathogen infections (Cramp et al., 2014; Kueneman et al., 2014; Rollins-Smith et al., 2011). In particular, a better understanding of the immunity attributes of amphibian skin has been the focus of a growing number of studies as it plays a critical role in host susceptibility to *B. dendrobatidis* skin colonisation and subsequent infection (Becker & Harris, 2010; Bletz et al., 2013; Brucker, Harris, et al., 2008; Harris et al., 2009; Holden et al., 2015; Kueneman et al., 2014; Lam et al., 2010; Lauer et al., 2007; Muletz et al., 2012; Shaw et al., 2014; Woodhams et al., 2007).

1.2. The role of cutaneous bacteria in amphibian immune function

Amphibian skin is a structurally complex, porous organ involved in a variety of vital physiological functions including cutaneous respiration, osmoregulation, chemical communication and immune defence (Kueneman et al., 2014). Mucous is a naturally occurring secretion associated with amphibian skin and is critical to its functioning. This mucous is rich in glycoproteins and mucopolysaccharides, providing a nourishing microhabitat in which microorganisms can reside (Bletz et al., 2013; Brizzi et al., 2002; Lauer et al., 2007). Microorganism-host interactions on amphibian skin include mutualistic, commensal and pathogenic relationships and thus play a critical role in amphibian health (Bletz et al., 2013; Cramp et al., 2014). Amphibian skin plays host to vital components of

amphibian acquired immune defence including mucosal antibodies (Kueneman et al., 2014; Lauer et al., 2007). However, as demonstrated by ineffective vaccination attempts, and the down-regulation of immune system genes, it is evident that adaptive immune response mechanisms are inadequate in protecting amphibians against pathogens such as *B. dendrobatidis* (Bletz et al., 2013; Rosenblum et al., 2009; Stice & Briggs, 2010). *B. dendrobatidis* antibodies have been found within cutaneous mucous in frog species showing continual decline due to *B. dendrobatidis* (Holden et al., 2015). Therefore, components of the innate immune system, including cutaneous antimicrobial peptides (AMP's), alkaloids and lysozymes, provide alternate pathways for the amphibian immune response (Holden et al., 2015; Kueneman et al., 2014; Lauer et al., 2007).

The production of AMP's from dermal granular glands varies across species and limits growth of cutaneous microbial species on amphibian skin (Holden et al., 2015). Amphibians support a diverse range of cutaneous bacteria, thus suggesting bacteria have developed defence mechanisms against AMP's and other components of amphibian immune defence via bacteria-host coevolution (Bletz et al., 2013; Holden et al., 2015; Lauer et al., 2007). However, amphibian pathogens such as *B. dendrobatidis* are also resistant to AMP's and alone these defences also appear inadequate in protection against infection (Lauer et al., 2007). Despite the ineffective nature of the innate and adaptive pathways discussed, many amphibian species demonstrate persistence with *B. dendrobatidis* whereas others do not. One possible explanation for this trend relates to the mutualistic properties of certain cutaneous bacteria (Becker et al., 2010; Lam et al., 2010; Lauer et al., 2007; Muletz et al., 2012; Woodhams et al., 2007).

Cutaneous bacteria species are known to be capable of influencing host susceptibility to pathogenic infections and thus form an additional component of amphibian innate defence through beneficial mutualistic associations (Lauer et al., 2007). As such, cutaneous bacterial communities have become a focal point of research targeting the suppression of *B. dendrobatidis* (Becker et al., 2010; Bletz et al., 2013; Brucker, Harris, et al., 2008; Harris et al., 2009; Holden et al., 2015; Lam et al., 2010; Lauer et al., 2007; Muletz et al., 2012; Shaw et al., 2014; Woodhams et al., 2007). Mutualistic cutaneous bacterial associations are well known in many vertebrate species, including humans, and perform a variety of critical roles (Gallo & Nakatsuji, 2011; Kueneman et al., 2014). For example, the

bacteria species *Staphylococcus epidermidis* prevents colonisation and overgrowth of pathogenic and opportunistic microbes on human skin (Gallo et al., 2011). Mechanisms by which beneficial bacteria provide host defence against amphibian pathogens may include the competition for space and other resources, alteration of the microhabitat on the skin, and the production of anti-fungal metabolites which are capable of inhibiting pathogen growth (Becker et al., 2010; Belden & Harris, 2007; Brucker, Harris, et al., 2008).

The production of anti-fungal metabolites forms the basis of an emerging body of culture-based research targeting the suppression of *B. dendrobatidis* through probiotic therapy (Becker et al., 2010; Bletz et al., 2013; Brucker et al., 2008; Harris et al., 2009; Lam et al., 2010; Lauer et al., 2007; Muletz et al., 2012; Shaw et al., 2014; Woodhams et al., 2007) . These compounds are capable of inhibiting the growth of *B. dendrobatidis* and are secreted by certain bacteria species. Studies have isolated bacteria species from amphibian skin which are capable of anti-fungal metabolite production and have shown these species enhance amphibian survival against *B. dendrobatidis* (Becker et al., 2009; Becker et al., 2010; Brucker, Harris, et al., 2008; Lam et al., 2010). For example, a recent study in mountain yellow-legged frogs (*Rana muscosa*) and redback salamanders (*Plethadon cinereus*) found that cutaneous bacteria isolated from these species were capable of inhibiting the growth of *B. dendrobatidis* in vitro as a result of anti-fungal metabolite production (Becker et al., 2010).

Targeting the fungal inhibitory properties of cutaneous bacteria is advantageous over typical amphibian immune response pathways given that *B. dendrobatidis* is expected to develop resistance to inhibitory mechanisms (Bletz et al., 2013). Bacteria have a much shorter evolutionary response time than host amphibians and thus can evolve mechanisms to maintain host protection as pathogens evolve (Bletz et al., 2013). This area of research explores the potential for inoculation of probiotic species on amphibian skin as a biological agent for pathogen control. Inoculating captive amphibians prior to release may potentially enhance post-release innate immune response and survival (Harris et al., 2009). An alternative method of probiotic treatment proposed is soil bio-augmentation with probiotic bacteria to provide an environmental reservoir of beneficial bacteria (Bletz et al., 2013; Muletz et al., 2012). However, prior to the introduction of probiotic preventative measures, thorough knowledge of species specific bacterial communities is required. Baseline data on

cutaneous bacteria communities of threatened amphibian species is minimal, and in Australia, almost non-existent (Antwis et al., 2014; Kueneman et al., 2014; Michaels et al., 2014; Shaw et al., 2014).

Amphibian skin hosts a diverse bacterial community that reflects a subset from their habitat (Culp et al., 2007). In order to develop a comprehensive understanding of cutaneous bacterial community attributes, it is critical to consider the environmental factors influencing which bacteria form the microbial community and determine bacteria-host dynamics. This will allow us to provide conditions within captivity which promote immune-enhancing mutualistic properties of cutaneous bacterial communities. Such influential factors have scarcely been examined, highlighting a key limitation to amphibian conservation-based knowledge. The primary determining factor may be the limited exposure to environmental bacteria reservoirs available in captivity, however, other factors are also likely to play a significant role (Antwis et al., 2014; Meyer et al., 2012; Michaels et al., 2014). For example, Meyer et al. (2012) found that environmental temperature influenced skin sloughing (shedding) frequency in cane toads, which in turn influenced bacterial abundance. Alternatively, Antwis et al. (2014) found that nutritional conditions, in particular carotenoid availability, significantly influenced community composition of cutaneous bacteria. These outcomes suggest that captive practices can directly and indirectly impact cutaneous bacterial communities. However, comprehensive investigation into amphibian species-specific effects is yet to be explored, and, for probiotic methods to be effective, a better understanding of captivity influences is required.

1.3. The Influence of dietary carotenoids on amphibian cutaneous bacteria

As outlined above, captive conditions can influence amphibian immune response (Antwis et al., 2014; Meyer et al., 2012; Michaels et al., 2014). In particular, nutritional conditions provided in amphibian *ex-situ* conservation programs may affect the composition of the cutaneous bacterial community, thus having crucial innate immunity implications (see section 1.2.). The influence of host diet on cutaneous bacterial communities may be a result of bacteria utilising nutrients directly for physiological functions such as growth, or the provision of a more suitable microhabitat for bacteria to reside through host utilisation of

dietary compounds (Antwis et al., 2014; Brizzi et al., 2002; Cogdell & Frank, 1987; Kirti et al., 2014; Liu et al., 2005).

One aspect of amphibian nutrition expected to influence health and immune function is the presence of dietary carotenoids. Carotenoids are a family of more than 600 compounds synthesised by photosynthetic organisms including plants, fungi and bacteria (Svensson & Wong, 2011; Vershinin, 1999). Vertebrates are unable to biosynthesize carotenoids *de-novo* and must acquire these compounds via dietary means (Alonso-Alvarez et al., 2004). Once acquired, carotenoids have been shown to exhibit efficient biological antioxidant activity, act as a precursor for vitamin A and influence vertebrate immune function (Vershinin, 1999). The potential beneficial effects of carotenoids on vertebrate physiological functions have been studied in a variety of species, including humans, birds, fish and amphibians (Blount & Matheson, 2006; Maiani et al., 2009; Martinez-Alvarez et al., 2005; Ogilvy et al., 2012b). For example, a study in greenfinches (Carduelis chloris) indicated dietary carotenoid supplementation significantly enhanced anti-body response (Aguilera & Amat, 2007). Similarly, carotenoids have been shown to enhance immune function in vertebrates, such as mammals, through enhancing proliferation and functioning of immune response components (Chew & Park, 2004). Carotenoids can also have indirect benefits resulting from trade-offs with other physiological functions, including the redirection of resources to development and growth (Alonso-Alvarez et al., 2008).

Carotenoids have also been shown to perform critical physiological roles in bacteria species such as the protection of cells against photo-oxidative damage and against hosts' reactive oxygen species within the digestive system (Cogdell et al., 1987; Fraser & Bramley, 2004; Kirti et al., 2014; Liu et al., 2005). Similar mechanisms may operate within cutaneous bacterial communities as has been suggested by Antwis et al. (2014). Within amphibian species, carotenoids show limited influence on health directly, however, they have been shown to enhance yellow, orange and red colouration in amphibian skin (Bailey, 2014; Ogilvy et al., 2012b). This suggests that carotenoids are incorporated into amphibian skin, and suggests that carotenoids influence skin structure and function. Given such effects, dietary carotenoids may not play a direct role in improving amphibian immune function, but may indirectly improve innate immune function through positive interactions with cutaneous bacteria. This possibility warrants investigation.

The influence of dietary carotenoids on amphibian cutaneous communities has only been investigated by one study. Recently, Antwis et al. (2014) examined the bacterial community associated with carotenoid-supplemented and un-supplemented red-eyed tree frogs (*Agalychnis callidryas*) and found that carotenoid supplementation significantly enhanced the bacterial community by increasing bacteria abundance and species richness. It is expected that carotenoid supplementation enhances the cutaneous bacterial community by: 1) bacteria utilising carotenoids directly for physiological functions or; 2) the provision of a more suitable microhabitat for bacteria to reside through host utilisation of dietary carotenoids (Antwis et al., 2014; Brizzi et al., 2002; Cogdell et al., 1987; Kirti et al., 2014; Liu et al., 2005).

Concepts linking cutaneous bacteria to the amphibian immune response are typically based on the assumption that an enhanced bacterial community is one with high diversity and abundance (Becker et al., 2010; Bletz et al., 2013; Brucker, Harris, et al., 2008; Harris et al., 2009; Holden et al., 2015; Lam et al., 2010; Lauer et al., 2007; Muletz et al., 2012; Shaw et al., 2014; Woodhams et al., 2007). This assumption stems from the notion that higher abundance is typically associated with high species richness, and high species richness is associated with increased stability and productivity (Belden et al., 2007; Eisenhauer et al., 2013; Johnson et al., 1996). The diversity-stability hypothesis and the productivity hypothesis form the basis of these arguments, each of which have tested a wide variety of ecological communities such as microbial communities (Eisenhauer et al., 2013). These hypotheses broadly link a more diverse community to improved stability and productivity respectively (Belden et al., 2007; Eisenhauer et al., 2013; Johnson et al., 1996; Matos et al., 2005; Van Elsas et al., 2012).

Given the cutaneous bacteria associated with amphibians previously examined differ vastly between species and across geographic locations, it is clear that influences may be species-specific and there may be no broad spectrum solution when relying on amphibian cutaneous bacteria to inhibit pathogen infection (Antwis et al., 2014; Kueneman et al., 2014; Michaels et al., 2014; Shaw et al., 2014). This warrants further investigation into the relationships between environmental conditions, microbial communities, immune function and potential probiotic therapies to remediate natural populations. Current research is biased towards a small number of species, including the American salamander species

Plethodon cinereus and *Hemidactylium scutatum* and the European and South American anuran species *Rana Mucosa*, *Agalychnis callidryas* and *Rana sphenocephala* (Antwis et al., 2014; Becker et al., 2010; Brucker, Harris, et al., 2008; Harris et al., 2009; Holden et al., 2015; Lam et al., 2010; Lauer et al., 2007; Michaels et al., 2014; Muletz et al., 2012; Shaw et al., 2014; Woodhams et al., 2007). Cutaneous bacteria research on Australian amphibians is minimal, and given the highly endemic nature of Australia's amphibian species, it is likely that bacterial communities on Australian species are significantly different to those that have been reported previously. Prior to the introduction of standardised husbandry protocols, and the implementation of probiotic preventative measures to enhance innate immune capabilities of amphibian species, thorough knowledge of species-specific bacterial communities is required. Additionally, in the context of the captive environment, an understanding of specific environmental variables which promote the immune-enhancing mutualistic properties of cutaneous bacterial communities is required before standardised conditions can be implemented.

In Australia, patterns of amphibian decline reflect the global trend with 48 species listed as threatened, 15 of which are critically endangered (IUCN, 2015). As listed on the IUCN Red List in 2002, the Southern Corroboree Frog (*Pseudophryne corroboree)* is one of Australia's most critically endangered species and thus forms the focus of this study (Hero et al., 2006; IUCN, 2015). *P. corroboree* is known to be particularly susceptible to *B. dendrobatidis* infection and populations are now almost solely maintained in *ex-situ* breeding programs (Brannelly et al., 2015; Hunter et al., 2010; OEH, 2012). These programs typically consist of relatively sterile husbandry conditions due to the substantial threat of *B. dendrobatidis* infection and thus environmental reservoirs of bacteria are limited. The Corroboree Frog has been the focus of a long term captive breeding program but species decline continues (OEH, 2012). As such, the urgent acquisition of species-related knowledge, in particular the influence of dietary conditions, is crucial in stimulating the success of captive breeding and reintroduction programs targeted at re-establishing natural populations of *P. corroboree*. The importance of captive breeding, reintroduction and the amelioration of environmental threats to *P. corroboree* is emphasised by its presence in the recovery objectives of the *P. corroboree* National Recovery Plan (OEH, 2012). Understanding how carotenoid availability affects anuran probiotic bacterial communities

may offer a potential strategy to enhance the post-release defence of individuals to deadly pathogens such as *B. dendrobatidis*.

The present study manipulates dietary carotenoid availability throughout juvenile and post-metamorphic life stages and applies bacteria culturing methods to compare the cutaneous bacterial community of supplemented and un-supplemented frogs within a captive colony. It is predicted that dietary carotenoid supplementation will enhance the cutaneous bacterial community of captive *P. corroboree,* based on the assumption that an enhanced community is one with high levels of species richness and abundance (refer to section 1.2.)*.* Outcomes of this study will contribute to a body of research targeting the development of standardised conditions which maximise the mutualistic properties of cutaneous bacterial communities, with the ultimate goal of improving the immunity of *P. corroboree* in captivity and post release.

1.4. Thesis Aims:

The aim of this study is to characterise the cutaneous bacterial community of the critically endangered Southern Corroboree Frog (*Pseudophryne corroboree*) in an ex-situ context and test the effect of dietary carotenoid supplementation on these cutaneous bacterial populations. Specifically, using the manipulation of dietary carotenoid availability throughout juvenile (tadpole) and post-metamorphic (frog) life stages within a captive colony of frogs, this project aims to:

1) Provide baseline community composition data for cutaneous bacterial communities (species presence, abundance and richness) in a captive *P. corroboree* population.
2) Determine if dietary carotenoid supplementation enhances abundance, species richness and affects overall community composition of cutaneous bacterial communities.

2. Methods:

2.1. Study species:

The Southern Corroboree frog (*Pseudophryne corroboree*) is a terrestrial anuran belonging to the family Myobatrichidae. This species is endemic to the sub-alpine regions of Kosciuszko National Park, Australia and is characterised by a distinct longitudinal black and yellow striped colouration pattern (Hero et al., 2006; Osbourne, 1991). This colour pattern is believed to indicate the presence of poisonous alkaloids to deter potential predators (Daly, 1998; Osbourne, 1991). *P. corroboree* is a small species (25-30mm snout vent length) that typically feed on algae and organic matter as tadpoles, and on ants and other small invertebrates as adults (Daly, 1998; Osbourne, 1991). The natural diet of *P. corroboree* outlined above contains dietary carotenoids (Osbourne, 1991) and thus it is assumed that under normal circumstances, *P. corroboree* would receive dietary carotenoids during all life stages.

Within this species' distribution, temperatures range from a mean monthly temperature of 16.3°C in summer (January) and -0.1°C in the peak of winter (July) (Bureau-of-Meteorology, 2015). Reproduction occurs in mid-late summer (January/February) whereby males construct terrestrial nests within sphagnum bogs, attracting female mates with the aid of an advertisement call. Attracted females will lay their eggs within the male's nest (Osbourne, 1991). The resulting fertilised eggs remain in diapause until autumn when rainfall-induced hypoxia triggers hatching (Osbourne, 1991). Tadpoles remain in shallow pools over winter and undergo metamorphosis during the next summer season. Adult *P. corroboree* enter a state of torpor at the onset of winter, thus undergoing slow development and reaching sexual maturity at 3-4 years of age (Osbourne, 1991). As listed on the IUCN Red List in 2002, *P. corroboree* is one of Australia's most critically endangered species (Hero et al., 2006; IUCN, 2015). Therefore, the acquisition of species-related knowledge, in particular the influence of dietary conditions, is crucial in stimulating the success of captive breeding and reintroduction programs targeted at re-establishing natural populations of *P. corroboree*. The importance of captive breeding, reintroduction and the amelioration of environmental threats to *P. corroboree*'s long term survival is emphasised in the recovery objectives of the P. corroboree National Recovery Plan 2014 (OEH, 2012).

2.2. Experimental animals:

Pseudophryne corroboree eggs (n = 44) were randomly selected from a total of 128 eggs generated from a captive colony housed at Melbourne Zoo, Australia. Fertile eggs were the result of pairings between 6 males and 12 females. These eggs were maintained at 5°C for a 3 month period on moist sphagnum moss (*Sphagnum cristatum*) prior to being transferred to the University of Wollongong on July 19th 2013. Upon arrival, eggs were flooded with reverse-osmosis water (R.O.) to stimulate hatching, which occurred over an 11 day period. Individuals were exposed to experimental dietary treatments throughout both juvenile and adult life-stages (refer to section 2.3.).

2.3. Experimental design

To examine the influence of dietary carotenoid supplementation on the cutaneous bacterial community of *P. corroboree,* 44 individuals were exposed to one of two dietary treatments with 22 individuals per treatment. Both tadpoles and frogs were randomly assigned to either a carotenoid-supplemented diet or a basal diet with no dietary carotenoid supplementation (Figure 1). In treatment 1 (hereafter referred to as 'un-supplemented'), frogs (n = 22) received a basal control diet with no dietary carotenoid supplementation throughout all life stages. In treatment 2 (hereafter referred to as 'carotenoid-supplemented'), frogs (n = 22) received dietary carotenoid supplementation throughout juvenile and post-metamorphic life stages. Details of dietary conditions for juveniles and post-metamorphic individuals can be found in section 2.4. The influence of dietary carotenoids has been shown to be gender specific (Ogilvy & Preziosi, 2012), but this could not be incorporated into the present study due to the age of experimental individuals (*P. corroboree* must be at least 4 years of age to be accurately sexed. Experimental individuals = 23 months at time of sampling) (Osborne, 1989). However, outcomes of recent research examining amphibian cutaneous bacteria have concluded that gender did not affect the influence of carotenoids on the diversity and abundance of amphibian cutaneous bacterial communities (Antwis et al., 2014).
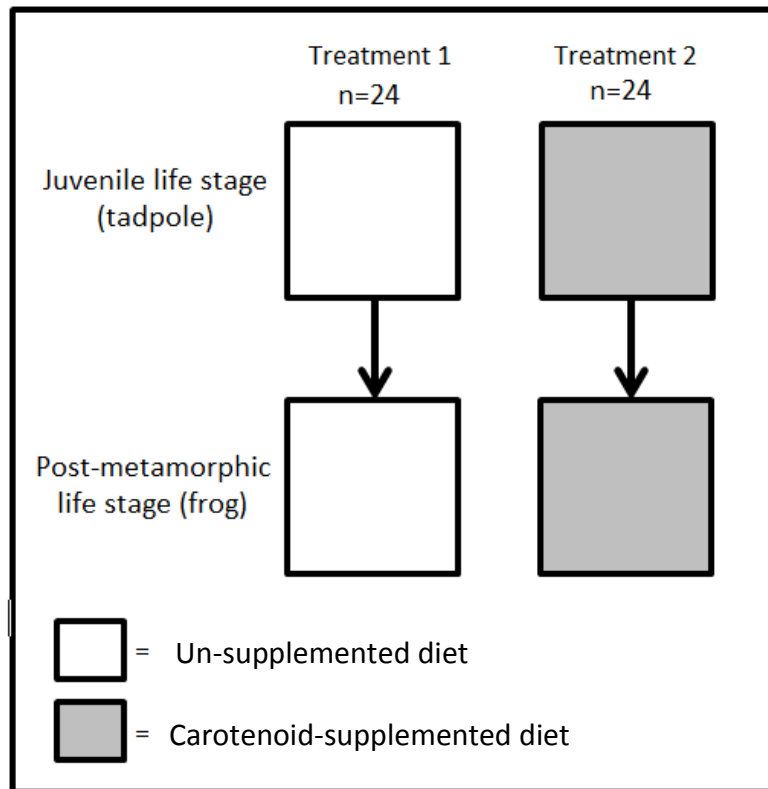
Figure 1. Diagrammatic representation of experimental dietary treatments

2.4. Frog husbandry

2.4.1. Juvenile husbandry

Immediately upon hatching, tadpoles (n = 44) were housed in cylindrical plastic containers (10.5cm H x 10cm D) containing 600ml of R.O. water. Tadpoles were maintained at 12°C (range = 11.4-12.9 °C) within a constant temperature room at the University of Wollongong Ecological Research Centre, Australia. Lighting conditions within the room consisted of artificial fluorescent lighting on an 11.5hr/12.5hr day-night cycle to simulate natural conditions (including 15mins twilight lighting at both dawn and dusk). Tadpoles also received 1hr/day (11:30 am-12:30pm) of UV-B light supplied by a Reptisun® 36" fluorescent strip bulb (Pet Pacific Pty Ltd, Sydney) suspended approximately 20cm above the tadpole containers to prevent developmental disorders known to be associated with UV-B deficiency in anurans (Lannoo, 2008).

Water fouling was prevented by a 50% water flush three times a week via an automated irrigation system (PIS Irrigation Systems, Australia) supplied with R.O. water (Sartoruis Stedim Biotech, Australia). In addition, excess food and excrement was siphoned from each container weekly using a syringe connected to aquarium tubing. Total ammonia levels (NH3 + NH4) were tested in three randomly selected containers per treatment every four weeks (Aqua One®, Australia) to ensure water quality remained at a healthy level. Ammonia levels typically ranged between 0.5-1mg L^{-1}, well within the safe zone for aquatic vertebrates. The above housing conditions were chosen after consultation with aquarists in the Herpetology Division of Taronga Zoo, Sydney.

Immediately after hatching, all tadpoles were fed dropwise on a basal diet of ground commercial fish flakes (25% san tropical, 75% flora vegetable flake; SERA®, Germany) suspended in 10ml of R.O. water for an average of 10±4 days due to asynchronous hatching. Feeding was conducted three times a week. At the commencement of the experimental period, tadpoles were randomly assigned to either a carotenoid-supplemented diet or a basal diet with no dietary carotenoid supplementation using a random numbers table.

The un-supplemented treatment consisted of a basal diet composed of 1.0g ground fish flakes (25% san tropical, 75% flora vegetable flake; SERA®, Germany) suspended in 10ml Reverse Osmosis (R.O.) water and fed three times a week ensuring food was always provided *ad libitum*. Basal diet mixtures were frozen at -20°C in 10ml syringes and thawed to room temperature on feeding days. Ensuring the mixture was homogenised via shaking. Individuals were fed 2 drops of the mixture (range = 0.0585g-0.0685g wet mass, 0.015g-0.018g dry mass) three times a week for the first 8 weeks and then fed four drops (range = 0.117g-0.137g wet mass, 0.03-0.036 dry mass) three times a week until metamorphosis occurred.

The carotenoid-supplemented diet consisted of the basal diet described above supplemented with 20mg carotenoid powder (per gram of food) (Superpig; Repashy®, USA). Similar doses (20mg) have been identified to have significant positive effects on survival, development and fitness determining traits in many vertebrates, in particular the Western Clawed frog (*Xenopus tropicalis*), thus justifying the identified dosage (Ogilvy & Preziosi, 2012).

2.4.2. Post-metamorphosis husbandry

Tadpole development was assessed every two days using Gosner staging tables (Gosner, 1960). At the onset of metamorphic climax, as represented by forelimb emergence (Gosner stage 42), individual containers were altered. Metamorph containers contained a sponge half submerged in 150mL R.O. water to enable the small frogs to crawl from the water during metamorphosis. Nutritional needs during this developmental stage are met by the reabsorption of the tail and body tissue catabolism, thus no food was provided (Wassersug & Wilbur, 1974). Once tail reabsorption had occurred (Gosner stage 46), individuals were fed an *ad libitum* diet of hatchling (first instar) live crickets (*Archea domestica*) twice a week. Post-metamorphic individuals remained in their metamorph container for 60±15 days prior to being transferred to experimental containers. Transfer occurred two weeks prior to the introduction of the adult (frog) experimental diets.

Cylindrical experimental containers (10.5cm H x 10cm D) consisted of a pebble base (2cm deep) overlain by approximately 5cm of sphagnum moss (*S.cristatum*) (Brunnings, Australia). A ring of small holes (1cm above the base) enabled a weekly flush of R.O. water (500ml) to remove excrement and uneaten food. All individuals were weighed 2 weeks prior to commencement of post-metamorphic experimental diets. Frog body mass did not differ between the two tadpole treatment groups prior to the introduction of post-metamorphic experimental diets (t-test: $t_{1, 47}$ = 0.842, p = 0.403).

The post-metamorphic basal diet consisted of an ad libitum diet of first instar crickets (*Archea domestica)* and adult fruit flies (*Drosophila melanogaster*) each provided once a week. Crickets were fed on granny smith apples and adult fruit flies (*D.melanogaster*) were provided with standard drosophila rearing mixture (Carolina Biological Supply Company, USA).

The post-metamorphic carotenoid-supplemented diet was composed of the above basal diet supplemented with dietary carotenoid mixture. This carotenoid supplementation consisted of gut loading crickets with carrot for 48hr prior to feeding and dusting with 1.0g carotenoid powder (Superpig; Rapashy®, USA). Fruit flies were fed on standard drosophila mixture containing an additional carotenoid mixture (ratio = 10:1 drosophila rearing mixture: carotenoid powder). This carotenoid supplementation provided 0.25mg g^{-1} of

carotenoids to the flies. The exact dose for frogs is unknown due to discrepancies in cricket and fly consumption. The carotenoid supplementation was chosen as a previous study had determined that 0.25mg g^{-1} dietary carotenoids significantly enhanced female growth, reproductive success and colour in red-eyed tree frogs (Ogilvy et al., 2012b). To prevent calcium deficiencies, crickets fed to both treatment groups were dusted with 0.2g calcium powder (Repti-cal; AristoPet, Australia) once a week (Lannoo, 2008). After four weeks of the experimental diet, fruit flies in each treatment were replaced with a second feed of first instar crickets. Fruit flies were initially supplied to ensure frogs had the opportunity to consume prey of an appropriate size.

2.5. Bacterial sample collection

In order to examine the influence of dietary carotenoid supplementation on the cutaneous bacterial community of *P. corroboree*, captive bred frogs were selected from a captive population within the University of Wollongong, Australia. Frogs had been exposed to one of two dietary treatments (n = 22 frogs per treatment) (Section 2.4.). Bacteria samples were collected weekly over a three week period on the 25/05/2015 (n = 5 per treatment), 01/06/2015 (n = 7 per treatment) and 08/06/2015 (n = 10 per treatment). There were no temporal effects of this sampling technique in cutaneous bacterial abundance or species richness observed (ANOVA: abundance: $F_{2, 41}$ = 1.20, P = 0.31; species richness: $F_{2, 41}$ = 0.69, P = 0.51). At the commencement of the experimental period, frogs were 23 months of age (17-18 months post-metamorphosis).

Frogs were removed from individual housing containers and initially weighed (to 0.01g) and photographed (Samsung ST66 16.1 megapixel camera, Samsung Group, South Korea) alongside a scale bar for later image analysis (IMAGE J) to obtain snout-vent length measurements (0.01mm). There was no significant difference in frog weight or length between the supplemented and non-supplemented treatments prior to swabbing (t-test: t_{43} = 1.30, P = 0.20; t_{43} = 0.99, P = 0.33) (n = 22 per treatment) Individuals were handled with separate sterile gloves (Skinshield powder free latex gloves, Livingstone International, Australia) and contacted surfaces were sterile to prevent cross contamination of cutaneous bacteria (Antwis et al., 2014; Lauer et al., 2007). Frogs were rinsed gently once with 30mL

sterile water to remove transient bacteria (Kueneman et al., 2014; Lauer et al., 2007; Shaw et al., 2014), ensuring minimal handling to prevent loss of cutaneous bacteria.

Cutaneous bacteria samples were collected using sterile transport swabs (Copan Transystem® Amies agar gel medium without charcoal, Copan Diagnostics, Inc., USA) using a swabbing procedure adapted from Shaw et al. (2014), Flechas et al., (2012) and Antwis et al. (2014). Frogs were swabbed (1 swab/frog) by running the natural fibre cotton tip across each of the following surfaces 3 times whilst rotating: dorsal (anterior to posterior), ventral (anterior to posterior), lateral (left and right sides), front legs from armpit to wrist (left and right sides), and back legs from groin to ankle (left and right sides) (Body regions are listed in order of swabbing) (Figure 2). Rotation of the swab throughout sample collection promoted even bacterial coverage on the swab. Labelled swabs were placed in the storage tube provided, which contained sterile transport media. Swabs in their storage tube were sealed in zip lock bags and stored on ice for 150 ± 30mins within a small esky (storage temp = 5.5 ± 0.6°C) while being transferred to the lab for processing. Particular care was taken to ensure the frogs were not harmed throughout the process and individuals were monitored post-swabbing for 4 weeks to ensure there were no signs of distress/injury as a result of the swabbing procedure.
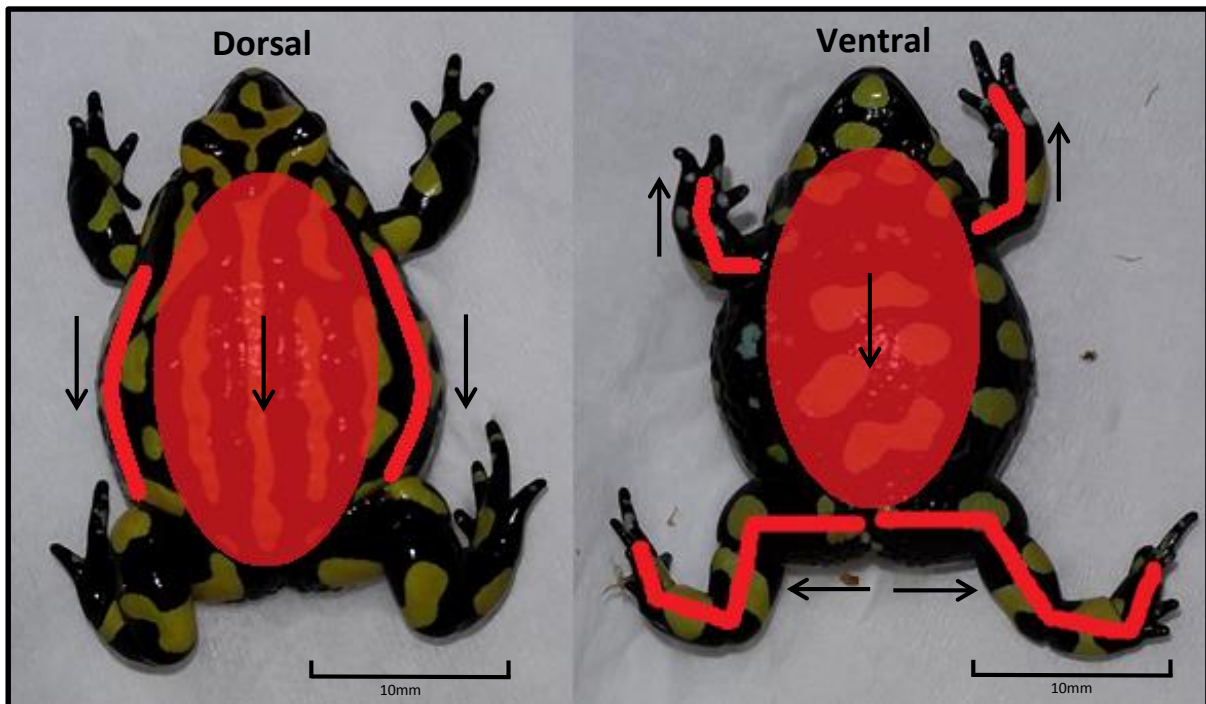
Figure 2. Cutaneous bacteria swabbing protocol on *P. corroboree* from dorsal and ventral perspectives. Areas in red represent body regions swabbed for bacteria. Frogs were swabbed (1 swab/frog) by running the natural fibre cotton tip across each of the following surfaces in triplicate whist rotating: dorsal (anterior to posterior), ventral (anterior to posterior), lateral (left and right sides), front legs from armpit to wrist (left and right sides), and back legs from groin to ankle (left and right sides). Body regions are listed in order of swabbing. Arrows represent direction of swabbing.

2.6. Bacterial culture:

Using aseptic techniques within a biosafety cabinet (Biological Safety Cabinet Class II, Email Westinghouse Pty Ltd, Australia), each swab was removed from the transport media and the swab tip cut off into a 1.5mL sterile Eppendorf tube (Eppendorf, Germany) containing 1000µL 0.9% sterile NaCl (Antwis et al., 2014; Nalven, 2013). Tubes containing swab tips were vortexed for 30 seconds to disassociate bacteria from the tip (Antwis et al., 2014). Swab tips were then removed from the tubes using sterile tweezers and solutions were vortexed for a further 5 seconds to ensure a homogenous bacterial suspension prior to

serial dilution and plating. A pilot study was performed to determine the optimum dilution for growth of *P. corroboree* cutaneous bacterial community (appendix 1). As indicated by this pilot study, dilutions of 10^1 (undiluted) and 10^{-1} (10 fold dilution) were most appropriate to obtain an intermediate amount of bacterial growth suitable for assessing bacterial community composition (CFU counts; Appendix 1). A 10^{-1} solution was prepared by pipetting 100µL of the initial solution into 900µL 0.9% sterile NaCl. All solutions were vortexed for a further 5 seconds immediately before plating.

Each swab was plated in triplicate (1×10^1 undiluted solution and 2×10^{-1} diluted solutions) by transferring 100µL aliquot of bacterial suspension to an LB (Luria Broth) nutrient agar media plate (100mm diameter) (AMRESCO®, USA) and spread evenly over the surface using a sterile plate spreader (Park et al., 2014). Plates were allowed to dry for 10mins within the biosafety cabinet prior to being inverted and sealed with parafilm® (Bemis Company, Inc., USA). Plates were incubated at 20°C for 168±24 hours to ensure sufficient time for development of colony morphology and where appropriate, they were transferred to 4°C to prevent overgrowth of fungi prior to species isolation. An incubation temperature of 20°C was selected based on the outcome of a pilot study testing optimum incubation temperature for growth of *P. corroboree* cutaneous bacterial community (Appendix 2). This is also the summer temperature in which the captive frogs are maintained.

Bacterial plate photographs were taken at 48±1, 72±1 and 96±1 hours (Samsung ST66 16.1 megapixel camera, Samsung Group, South Korea) with the aid of a backlight and including scale and label (frog ID, diet treatment, incubation time, incubation temperature and date) (figure 3). As indicated by the pilot study, an accurate CFU count could be obtained by 72hrs while maintaining maximum time efficiency (Appendix 2). Photographs taken at 72±1 hours were analysed using ImageJ (National Institute of Health, USA) to obtain total abundance counts of CFU's (Colony Forming Units) (figure 3). The most appropriate plate from each triplicate was selected for analysis on the basis of total CFU range within 25-300 CFU (Sutton, 2011). A final result of CFU/mL was calculated using the dilution factor of each plate.
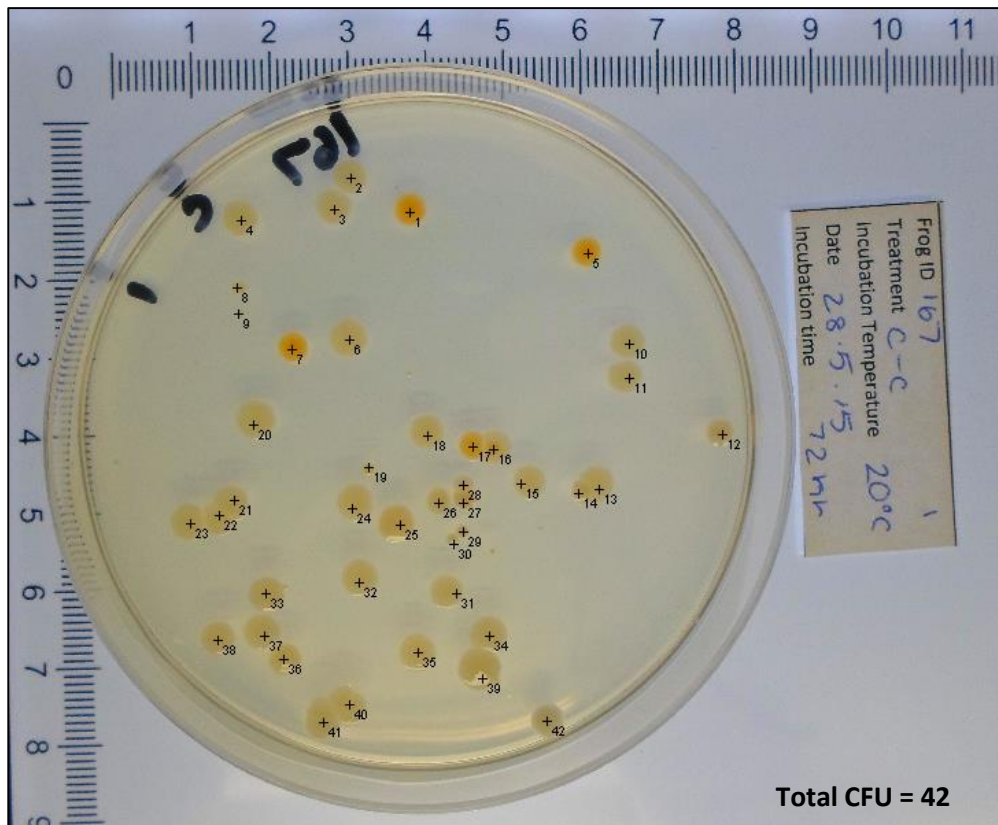
Figure 3. Bacteria culture plate image illustrating method for analysing plate images to obtain Colony Forming Unit (CFU) counts. Cross pointers represent CFU counts analysed using ImageJ (National Institute of Health, USA). Total CFU represents the absolute number of CFU on the plate (not accounting for dilution factor of the plate).

2.7. Bacteria Colony Isolation

After incubation at 20°C for 168±24 hours, all morphologically distinct colonies within each set of three dilution plates were allocated an isolate ID number and transferred to a separate LB nutrient agar plate using standard 16 streak method to obtain pure cultures (Washington et al., 2006). Details of each isolate were recorded (colour, form, margin, texture) (Washington et al., 2006). A species richness count for each individual frog was determined. Isolate plates were incubated at 20°C for 168±24 hours to ensure distinct colony morphological characteristics were evident. Isolate plates were compared across individuals and morphologically distinct isolate plates were assigned a Bacteria Morphotype ID number. After the initial incubation period, pure culture isolate plates were transferred to storage at 4°C. One representative from each Morphotype ID number was then processed for species Identification.

2.8. Bacteria Identification

Bacterial Species identification was conducted using API® Biomérieux 20E™ Identification system (Biomérieux, USA). This system consists of a series of 20 microtube tests and 7 additional tests conducted as per manufacturer's instructions (API® Biomérieux 20E™, USA). Prior to identification, pure isolates were re-plated on LB Nutrient agar and MacKonkey agar using a 16 streak pattern and incubated for 24 hour at 37°C. The combination of test outcomes from the test strip results in the formation of a 7-9 digit code which was entered into apiweb™ identification software (Biomérieux, USA). This software provides an output giving listing the most probably species identity. API® Biomérieux 20E™ bacterial identification is limited to *Enterobacteriaceae* and non-fastidious gram-negative species and thus all species could not be identified. Species that could not be identified using this system (n = 8) were sent to Taronga Zoo for lab analysis using Matrix Assisted Laser Desorption/ Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF) (Bruker Daltonik MALDI Biotyper, Bruker Biosciences, Victoria). Species that could not be identified using either system were assigned an unidentified species letter accompanied by a detailed morphotype description (appendix 3).

2.9. Data analysis

Counts of bacterial abundance (CFU) were multiplied by the dilution factor associated with each individual plate to obtain a standardised measure in CFU/mL of suspension solution. Average bacterial abundance (CFU/ml) was calculated for each treatment and analysed using t-test analysis in JMP 10® to determine the effect of carotenoid supplementation on amphibian cutaneous bacteria abundance. In order to determine the effect of carotenoid supplementation on amphibian cutaneous bacteria species richness, counts were compiled for each treatment and treatment averages were calculated. Species richness results were also analysed using t-test analysis in JMP 10®. Prior to this analysis, the effect of bacterial abundance on bacterial species richness was quantified using regression analysis. This was done to determine whether bacterial species richness could be assessed independently or whether it was positively correlated with abundance and thus simply a product of bacterial abundance. Outcomes of this analysis indicated that the t-test analysis described above was

suitable given no significant correlation between abundance and species richness (regression: $F_{1, 42}$ = 0.0091, P = 0.9243, r^2 = 0.0002). The absence of an expected positive correlation appeared to be attributed to the dominance of particular single species on plates with high CFU counts, thus an increase in CFU did not necessarily mean an increase in species richness.

Prior to running the t-test models, the assumptions of analysis were tested using Shapiro-Wilk's normality test and the Levene's test for homoscedasticity. Where required, response variables were log-transformed to ensure data was normally distributed. This was only required for bacterial count data (CFU). As indicated by regression analysis using JMP10®, frog mass (g) did not have a significant effect on total bacterial abundance or bacterial species richness and thus could be excluded from subsequent analysis (regression: abundance: $F_{1, 42}$ = 0.0120, P = 0.9133 r^2 = 0.000261; species richness: $F_{1, 42}$ = 1.1757, P = 0.2839 r^2 = 0.000261). Likewise, size of individual (snout-vent length in mm) was excluded from subsequent analysis as there was no effect of size on total bacterial abundance or bacterial species richness (abundance: $F_{1, 42}$ = 1.2027, P = 0.2785 r^2 = 0.025479; species richness: $F_{1, 42}$ = 0.0120, P = 0.9133 r^2 = 0.025479). As such, mass and size could be excluded from analysis models and thus t-tests were suitable as outlined above.

A bacteria species presence/absence data set was created to allow for a comparison of overall bacterial community composition between dietary treatments, which was performed using multivariate analysis in PRIMER 7. Initially, a Bray Curtis similarity index and Multi-dimensional scaling (nMDS) ordination was produced to allow for a visual representation of similarities between each frog's cutaneous bacterial community. This approach allows for a comparison of treatment-related patterns before proceeding with further analysis. Differences in overall community composition of cutaneous bacteria between treatments was analysed using PERMANOVA. SIMPER analysis was subsequently used to determine which bacterial species were most influential in determining differences between bacterial species assemblages of frogs in each diet treatment. These are nonparametric analyses and thus the assumption of normality need not be met. Data was from random, independent samples. Additionally, bacterial species assemblage data was compiled to produce a list of species present within the different dietary treatments.

3. Results:

3.1. Effect of dietary carotenoid supplementation on total bacterial abundance

A range of 370-42,100 culturable CFU/mL (MEAN ± SEM = 8,604 ± 2,276) were obtained from individuals receiving dietary carotenoid supplementation compared with a range of 160-9,700 culturable CFU/mL (MEAN ± SEM 2,119 ± 526.8) from individuals fed on a carotenoid-free diet. Dietary carotenoid supplementation had a significant effect on total cutaneous bacterial abundance (CFU/mL) (t-test: t_{43} = 3.16412, P = 0.0014) (figure 4). Individuals fed on a carotenoid supplemented diet supported a significantly greater bacterial abundance than individuals fed on an un-supplemented diet (figure 4).
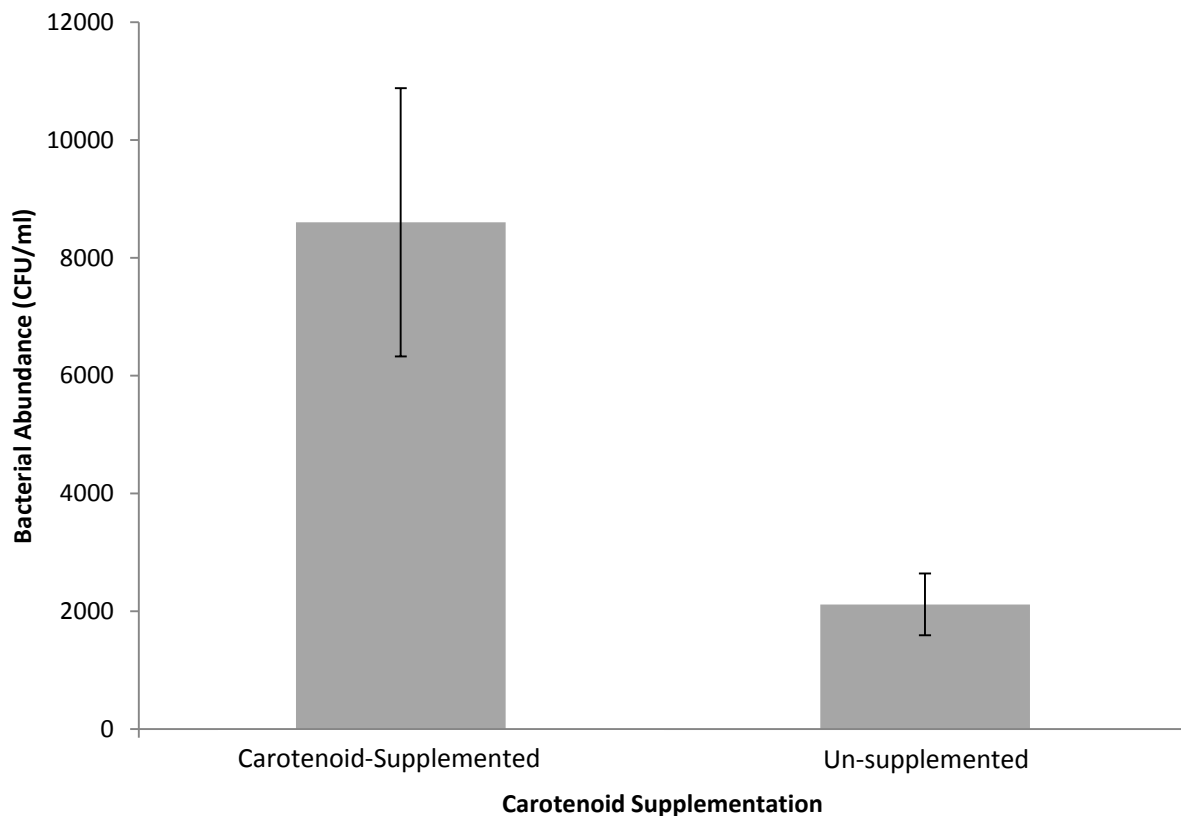
Figure 4. Average cutaneous bacterial abundance (Colony forming units (CFU) per mL) ± SEM isolated from the skin of *P. corroboree* fed on a carotenoid-supplemented and an unsupplemented diet (n = 22 per treatment). CFU/mL is equivalent to total culturable CFU obtained from sampling of each individual.

3.2. Effect of dietary carotenoid supplementation on bacterial species richness

The number of bacterial species isolated per individual ranged from 7-12 species (MEAN ± SEM = 8.83 ± 0.32) for frogs receiving dietary carotenoid supplementation and 4-11 species (MEAN ± SEM = 6.79 ± 0.36) for frogs fed an un-supplemented diet (figure 5). Dietary carotenoid supplementation had a significant effect on bacterial species richness (t-test: t_{43} = 4.25299, P = 0.0001) (figure 5). Individuals fed on a carotenoid-supplemented diet supported a greater number of cutaneous bacteria species than individuals fed on an un-supplemented diet (figure 5).
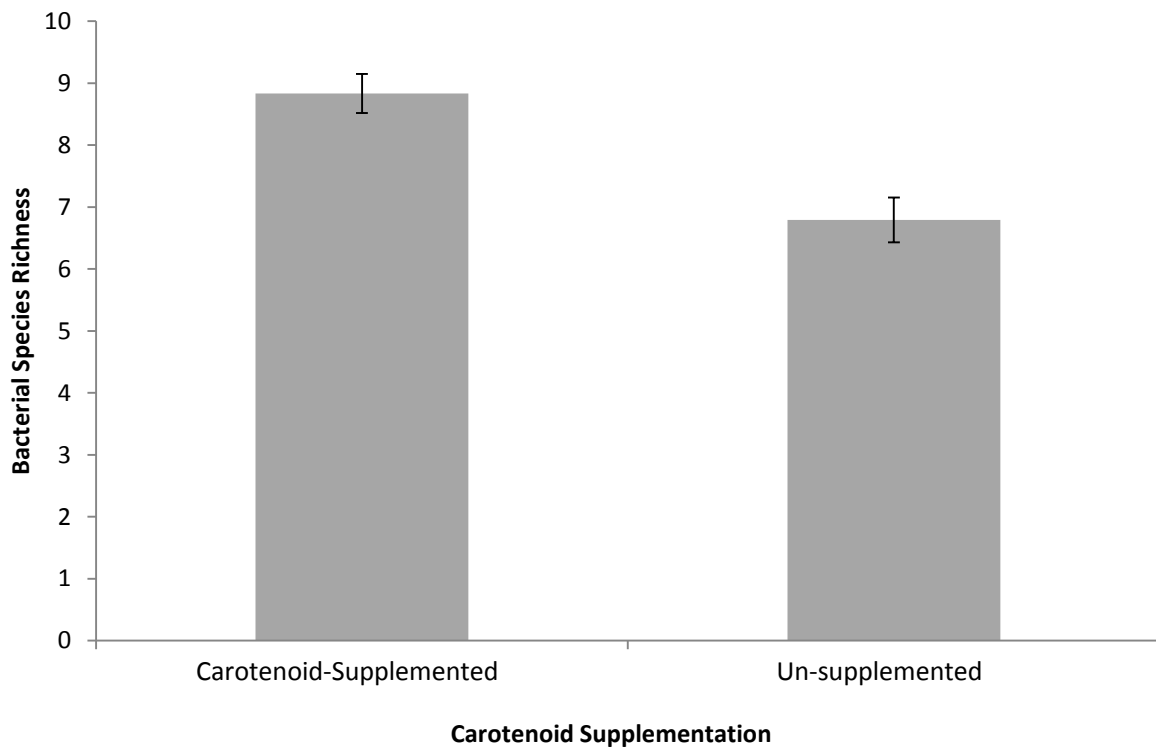
Figure 5. Average number of bacterial species (bacterial species richness) ± SEM isolated from the skin of *P. corroboree* fed on a carotenoid-supplemented and an un-supplemented diet (n = 22 per treatment).

3.3. Effect of dietary carotenoid supplementation on bacterial community composition

There was a total of twenty-three bacterial morphotypes isolated from captive *P. corroboree* (table 1; Appendix 4)*. Twenty-two of these bacterial morphotypes were present on carotenoid-supplemented *P. corroboree* and twenty bacterial morphotypes were present on *P. corroboree* fed an un-supplemented diet. Nineteen of these bacterial morphotypes were found in both carotenoid-supplemented and un-supplemented treatments. In total, 83% of bacteria species were found in both treatments. Three bacteria species (*Escherichia coli, Acinetobacter calcoaceticus* and Unidentified C), were isolated from the skin of carotenoid-supplemented individuals but were not present on any un-supplemented individuals. One species, *Variovorax paradoxus*, was only present on the skin of un-supplemented individuals (table 1). Five bacterial morphotypes could not be identified using API20E or MALDI-TOF (Appendix 3).

Overall community composition of cutaneous bacteria (species assemblages) was significantly different between *P. corroboree* frogs fed on a carotenoid-supplemented diet and those fed a diet free from carotenoid supplementation (PERMANOVA: Pseudo-$F_{1, 46}$ = 3.6754, P (perm) = 0.003). However, treatment groups had a relatively low average dissimilarity of 48.92% and a Multi-Dimensional Scaling plot (MDS) could not appropriately arrange individuals on a 2D or 3D plane to depict relative similarities (stress value = 0.26, 0.18 respectively) (figure 6). *Pseudomonas luteola* contributed most to species assemblage differences (7.53% contribution of total dissimilarity) and was more common within the dietary carotenoid supplementation treatment (χ^2_1 = 29.673, P < 0.0001). Another four species that were significantly more common within the dietary carotenoid supplementation treatment group were: *Ewingella americana* (%contribution = 6.76, χ^2_1 = 25.259, P < 0.0001); Unidentified A (%contribution = 6.39, χ^2_1 = 13.032, P = 0.0003); *Rhodococcus erythopolis* (%contribution = 6.25, χ^2_1 = 20.051, P < 0.0001); and *Serratia marcescens* (%contribution = 5.76, χ^2_1 = 33.122, P < 0.0001). No species occurred more commonly within the un-supplemented treatment. *Stenotrophomonas maltophilia* was the most commonly occurring species, being present on all individuals and contributing 19.53% to total treatment similarity.

Table 1: Comparison of bacteria species isolated from captive *P. corroboree* fed on a carotenoid-supplemented (n=22) diet and an un-supplemented diet (n=22).

Family	Species (APIWeb % match)	Percentage occurrence of bacteria species in captive *P. corroboree*. (The number in brackets represents the number frogs with bacteria species present)	
		Carotenoid Supplemented Frogs	Un-supplemented Frogs
Aeromonadaceae	*Aeromona hydrophila* (80%)	18.2 (4)	36.4 (8)
Comamonadaceae	*Variovorax paradoxus**	59.1 (13)	45.5 (10)
Enterobacteriaceae	*Citrobacter braakii* (99.3)	40.9(9)	54.5 (12)
Enterobacteriaceae	*Citrobacter freundii* (99.8%)	9.1 (2)	18.2 (4)
Enterobacteriaceae	*Escherichia coli* (87%)	4.5 (1)	0 (0)
Enterobacteriaceae	*Ewingella americana**	45.5 (10)	13.6 (3)
Enterobacteriaceae	*Klebsiella oxytoca* (99%)	36.4 (8)	27.3 (6)
Enterobacteriaceae	*Klebsiella pneumoniae spp* (99.9%)	18.2 (4)	4.5 (1)
Enterobacteriaceae	*Morganella morganii* (98%)	72.7 (16)	77.3 (17)
Enterobacteriaceae	*Proteus mirabilis* (89.9%)	59.1 (13)	50 (11)
Enterobacteriaceae	*Serratia marcescens* (96%)	4.5 (1)	9.1 (2)
Enterobacteriaceae	*Serratia spp* (92%)	90.9 (20)	54.5 (12)
Moraxellaceae	*Acinetobacter calcoaceticus* (99%)	4.5 (1)	0 (0)
Nocardiaceae	*Rhodococcus erythopolis**	45.5 (10)	18.2 (4)
Pasteurellaceae	*Pasteurella pneumotropica* (87%)	9.1 (2)	4.5 (1)
Pseudomonadaceae	*Pseudomonas luteola* (91%)	68.2 (15)	27.3 (6)
Pseudomonadaceae	*Pseudomonas oryzihabitans* (87.3%)	4.5 (1)	9.1 (2)
Xanthomonadaceae	*Stenotrophomonas maltophilia* (99.3%)	100 (22)	100 (22)
	Unidentified A	54.5 (12)	31.8 (7)
	Unidentified B	63.6 (14)	59.1 (13)
	Unidentified C	36.4 (8)	0 (0)
	Unidentified D	18.2 (4)	13.6 (3)
	Unidentified E	0 (0)	31.8 (7)

*Species without API% Match were identified using MALDI-TOF
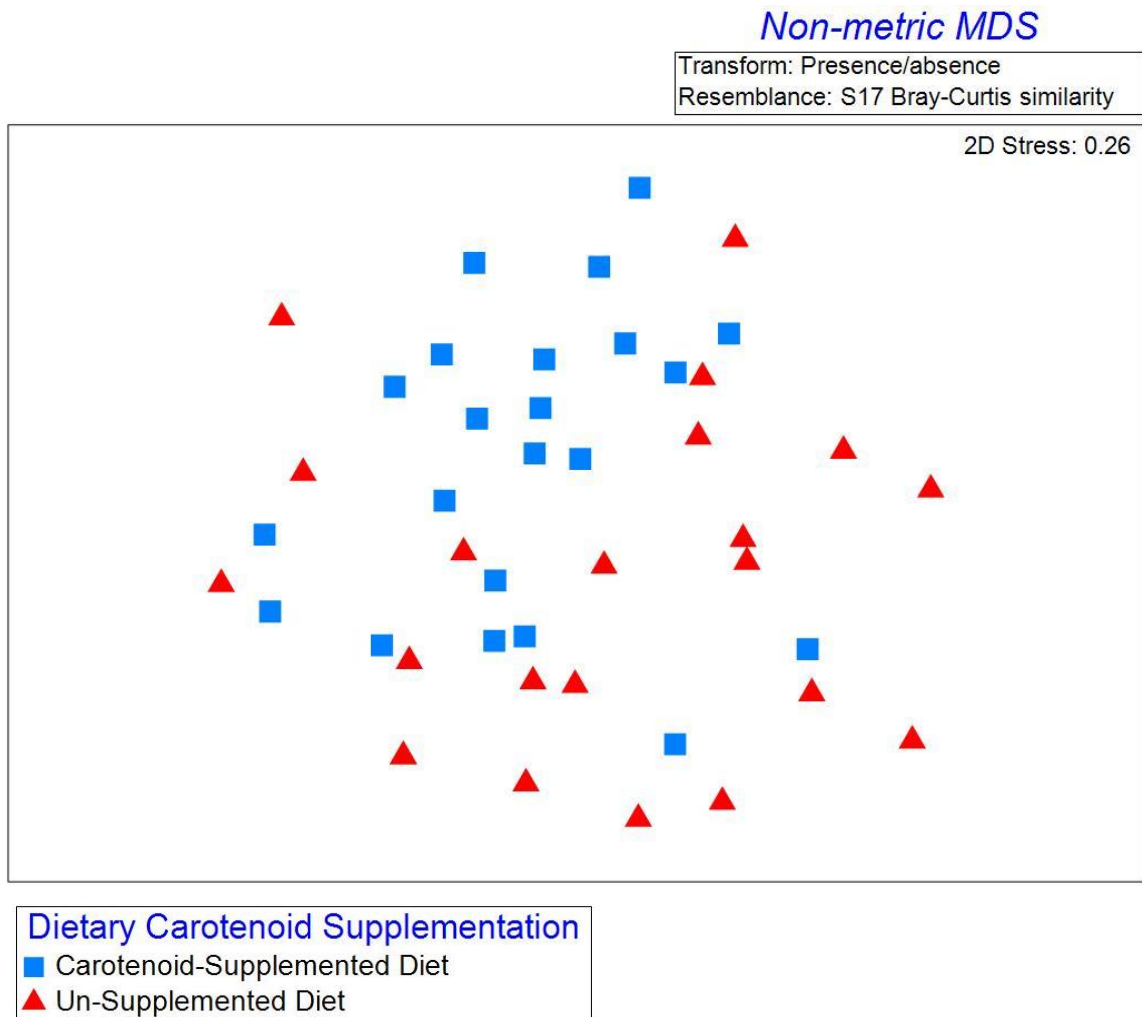
Figure 6. Multi-Dimensional Scaling (nMDS) depicting relative similarities of bacteria species assemblages of *P. corroboree*. Each point represents individual frogs and their associated bacteria species assemblage. Points have been arranged such that the distances between them represent their relative similarity (n = 22 per treatment).

4. Discussion

The aim of this study was to characterise the cutaneous bacterial community of the critically endangered Southern Corroboree Frog (*Pseudophryne corroboree*) in an ex-situ context, and to test the effect of dietary carotenoid supplementation on these cutaneous bacterial populations. Dietary carotenoid availability was manipulated throughout juvenile and post-metamorphic life stages to determine whether dietary carotenoid supplementation enhanced abundance, species richness and affected overall community composition of cutaneous bacterial communities. As predicted, the provision of dietary carotenoids was associated with a significantly greater abundance of cutaneous bacteria. Carotenoid supplementation was also associated with a greater species richness of bacteria species on the skin of individual frogs. Bacterial species assemblages (community composition) differed significantly between individuals fed on a carotenoid-supplemented diet and individuals fed a diet free from carotenoids. However, despite significant differences, species assemblages between treatment groups were only 48.92% dissimilar, suggesting that group differences were relatively small. The absolute numbers of species isolated from carotenoid-supplemented and un-supplemented treatment groups were also similar (22 and 20 respectively), 83% of which were consistent across both treatment groups. Based on the assumption that an optimal bacteria community is one with high levels of species richness and abundance (Eisenhauer et al., 2013), the results obtained support our initial hypothesis that dietary carotenoid supplementation enhances the cutaneous bacterial community of captive *P. corroboree*.

There are several possible explanations as to why carotenoid supplementation enhanced the cutaneous bacterial community of *P. corroboree*. An increased species richness and abundance of bacteria on frogs from the carotenoid-supplemented treatment suggests the presence of carotenoids within the host's diet improves reproduction, growth and/or survival capabilities of certain bacteria on the host's skin (Antwis et al., 2014; Kirti et al., 2014). Such beneficial effects of carotenoids have been demonstrated in a variety of vertebrate species, including humans, birds, fish and amphibians (Blount et al., 2006; Maiani et al., 2009; Martinez-Alvarez et al., 2005; Ogilvy et al., 2012b). Carotenoids have been shown to be invested in a variety of vertebrate physiological functions including immunity

roles as antioxidants and free-radical scavengers, as well as in the development of signalling systems (colour) and visual systems (Bailey, 2014; Goodwin, 1986; McGraw & Hill, 2006; Vershinin, 1999). For example, recent research in *P. corroboree* has shown that dietary carotenoid supplementation improves skin colouration (Bailey, 2014). However, within amphibian species, there is a growing body of evidence indicating that carotenoids are not critical for survival, growth and body condition (Butler & McGraw, 2012; Cothran et al., 2015; Ogilvy & Preziosi, 2012; Rahman et al., 2013). Recent research into the effects of dietary carotenoid supplementation on *P. corroboree* performance found that carotenoid supplementation had limited influence on foraging performance, anti-predatory behaviour, survival, growth rate, body size and body condition, but significantly enhanced skin colouration (Bailey, 2014; McInerney, 2014). Based on these findings, it appears that carotenoid supplementation may not play a direct role in enhancing physiological traits of *P. corroboree,* but may improve amphibian immune function through positive effects on cutaneous bacteria populations (Antwis et al., 2015; Becker et al., 2010; Lam et al., 2010; Woodhams et al., 2007).

Like many other species, bacteria utilise carotenoids in physiological functions, suggesting that the improved availability of carotenoids within the cutaneous bacterial species may enhance the effectiveness of these functions. For example, some bacteria species utilise carotenoids to protect cells against photo-oxidative damage and some are capable of carotenoid biosynthesis (Cogdell et al., 1987; Kirti et al., 2014; Liu et al., 2005). One such explanation for the trends observed in this study relates to the physiological role carotenoids play within bacteria. Upon ingestion by a host species, bacteria cells have been shown to utilise the antioxidant properties of carotenoids in order to protect themselves against damage by reactive oxygen species within the host (Fraser et al., 2004; Liu et al., 2005). In recent research, bacteria species isolated from the skin and gut of two amphibian species (*P. cinereus* and *R. pipiens*) have been shown to be similar, indicating that these bacterial communities are of similar origin (Wiggins et al., 2011). Given that amphibians are known to periodically shed and ingest their skin (sloughing), carotenoid-mediated survival of bacteria within the host amphibian may play a significant role in establishing/regulating the cutaneous bacterial community (Antwis et al., 2015; Cramp et al., 2014; Weldon et al., 1993). It is also likely that this mechanism is particularly important in captive frog

populations where there is limited access to environmental reservoirs of a wide range of bacteria species (Becker et al., 2014). While these mechanisms may cause the patterns observed in the present study, they are yet to be examined in the context of enhancing amphibian immune function. Therefore, future work could examine relationships between gut and skin bacteria within *P. corroboree* to assess similarities between these communities and examine the influence of these relationships on frog immune response, and general health.

Bacterial-mucous interactions on the skin of the amphibian host provide an alternative explanation for why carotenoid supplementation enhances the cutaneous bacterial community associated with *P. corroboree*. Mucous is a naturally occurring secretion associated with amphibian skin that helps maintain the physiological functions associated with the skin, such as respiration and osmoregulation (Kueneman et al., 2014). Amphibian mucous can also provide a physical barrier to infection (Brizzi et al., 2002; Kueneman et al., 2014). This mucous is likely to be of nutritional value to the cutaneous bacterial community residing on the skin, and dietary attributes of the amphibian host are likely to influence the production and composition of this mucous (Brizzi et al., 2002; Lauer et al., 2007). Very little is known about the impact of carotenoids on amphibian skin mucous secretions, however, effects have been observed in the glandular epithelium of amphibian tongues (Miller et al., 2001; Ogilvy et al., 2012b; Pessier, 2002). For example, vitamin A deficiencies have been associated with keratinisation of the glandular epithelium on amphibian tongues resulting in a reduction in mucous production, a condition called Hypovitaminosis A (Miller et al., 2001; Ogilvy et al., 2012b; Pessier, 2002). Given that carotenoids are a precursor for vitamin A, it is possible that similar mechanisms may occur in the glandular tissues of amphibian skin. This may result in decreased mucous production in individuals not receiving dietary carotenoid supplementation, thus resulting in a sub-optimal microhabitat in which cutaneous bacteria reside. Given limited knowledge in this area, future research would benefit by focusing on the analysis of amphibian skin mucous to determine the nutritional content. This would allow for the determination of whether carotenoid supplementation within the host amphibian alters mucosal secretion composition in a way that benefits cutaneous bacteria communities.

The above explanations are based on the assumption that enhanced abundance and species richness within a community are associated with increased productivity and stability, as suggested by the diversity-stability hypothesis and the productivity hypothesis (Belden et al., 2007; Eisenhauer et al., 2013; Johnson et al., 1996). Improved productivity and stability has been linked to enhanced resilience to perturbations and stresses as well as an improved response to environmental changes, such as those experienced during the transfer of individuals from captivity to the wild (Belden et al., 2007; Eisenhauer et al., 2013; Johnson et al., 1996; Matos et al., 2005; Miller et al., 2001; Van Elsas et al., 2012) . These mechanisms have been shown to operate within various systems, including microbial communities, and thus, it may be inferred that they may also operate within amphibian cutaneous bacteria communities (Eisenhauer et al., 2013; Matos et al., 2005; Miller et al., 2001; Van Elsas et al., 2012). Enhanced species richness and abundance within these cutaneous bacteria communities may make the frog more resilient to environmental changes such as those experienced after release from captivity (Antwis et al., 2014; Kueneman et al., 2014). It also may make them more resilient to pathogen stresses (such as *B. dendrobatidis*) within captivity, as well as post-release (Longo et al., 2015). Therefore, given that carotenoid supplemented *P. corroboree* individuals support an enhanced bacterial community, this community may be more proficient at sustaining a healthy bacterial community throughout a variety of perturbations. In turn, this may lower susceptibility to pathogen infection of the host amphibian within captivity and post-release (Antwis et al., 2014; Becker et al., 2014; Becker et al., 2015; Bletz et al., 2013; Lam et al., 2010; Longo et al., 2015; Shaw et al., 2014).

A high density of bacteria is required to allow for successful cell communication, a process known as quorum sensing (Boyen et al., 2009; Li & Tian, 2012; Salmond et al., 1995). Quorum sensing is the process whereby bacteria cells produce signal molecules to allow for communication between cells enabling community-wide coordinated physiological processes/responses (e.g. antifungal metabolite production) (Boyen et al., 2009; Li & Tian, 2012; Salmond et al., 1995). Enhanced richness of bacteria on the skin of *P. corroboree* is also likely to be associated with increased competition for limited space, and a reduced likelihood of the presence of a vacant ecological niche susceptible to pathogen establishment (Bessen et al., 2005; De Boer et al., 2005; Herbold & Moyle, 1986;

Wiedenbeck & Cohan, 2011). Enhanced species richness also increases the likelihood of the host amphibian possessing bacteria species capable of inhibiting the infection of pathogens such as chytrid fungus (*B. dendrobatidis*) (Antwis et al., 2014; Lam et al., 2010; Shaw et al., 2014).

Cutaneous bacteria inhibitory mechanisms have been shown in a variety of amphibian species and are typically an outcome of antifungal metabolite production by specific bacteria species (Becker et al., 2010; Brucker, Baylor, et al., 2008; Brucker, Harris, et al., 2008; Lam et al., 2010; Woodhams et al., 2007). For example, a recent study in redback salamanders (*Plethadon cinereus*) found that the cutaneous bacteria species *Janthinobacterium lividum* isolated from these salamanders was capable of inhibiting the growth of *B. dendrobatidis in vitro* via the production of violacein, an anti-fungal metabolite (Becker et al., 2009). Some frog species such as *Rana spenocephala* are capable of anti-microbial peptide (AMP) production via granular skin glands, which has also been shown to inhibit pathogens such as *B. dendrobatidis* (Holden et al., 2015; Pessier, 2002). However, given the current decline of *P. corroboree* in the wild has been attributed to B. dendrobatidis, it is likely that *P. corroboree* is not capable of producing *B. dendrobatidis* inhibitory AMPS, and thus, may possibly rely on antifungal metabolite production by bacteria as an alternative immune strategy (Becker et al., 2010; Brucker, Baylor, et al., 2008; Brucker, Harris, et al., 2008; Holden et al., 2015; Hunter et al., 2010; Lam et al., 2010; Pessier, 2002). A recent study in *P. cinereus* also highlighted the importance of high bacterial diversity and species interactions by demonstrating that co-cultures of bacterial species were more proficient at inhibiting the growth of *B. dendrobatidis* when compared to single species cultures (Loudon et al., 2014). It is not yet known whether bacteria species identified in the present study, which occur on *P. corroboree,* are capable of antifungal metabolite production. Therefore, future research should incorporate inhibition assays using bacteria and pathogens such as *B. dendrobatidis* to develop an understanding of cutaneous bacteria species ability to protect against pathogens.

The present study contributes to an emerging body of knowledge investigating the effects of *ex situ* captive conditions on amphibian cutaneous bacteria (Antwis et al., 2014; Becker et al., 2014; Loudon et al., 2014; Meyer et al., 2012; Michaels et al., 2014). Previous studies in other frog species (*Agalychnis callidryas* and *Atelopus zeteki*) have looked at the

overall influence of captivity on amphibian cutaneous bacteria through bacteria community comparisons with wild populations and have found significant effects (Antwis et al., 2014; Becker et al., 2014). However, there remains a limited understanding about the influence of dietary carotenoids on these cutaneous bacterial communities, with only one study investigating these effects (Antwis et al., 2014). Antwis et al. (2014) showed that dietary carotenoid supplementation enhanced cutaneous bacterial communities in *A. callidryas*. Results of the present study were consistent with Antwis et al. (2014) as they also found an enhanced cutaneous bacterial community associated with carotenoid-supplemented individuals. Despite *A. callidryas* being a much larger species than *P. corroboree*, bacterial species richness and abundance were similar across studies, with each study demonstrating higher outcomes in carotenoid-supplemented individuals. Interestingly, despite significant size differences, *P. corroboree* had substantially greater bacterial abundance, suggesting that it may provide a more optimal microhabitat for bacteria to reside (Antwis et al., 2014). However, these differences may simply be attributed to differences in sampling methodology such as swabbing technique, swab storage and culturing techniques. Nevertheless, these concordant outcomes further support the concept that dietary conditions experienced in captivity may affect the immunity and health of individuals while in captivity and post-release.

However, it is important to note that the carotenoid supplementation results of the present study (and of Antwis et al. 2014) are specific to captive individuals, and thus, the patterns observed in wild individuals may be different. These differences are likely to be primarily a result of individuals having access to environmental reservoirs of bacteria in the wild (Becker et al., 2014). Conditions experienced in the wild are likely to fluctuate substantially in comparison to stable captive conditions, therefore, further work is required to compare cutaneous bacteria communities between wild and captive *P. corroboree*. This would allow for the evaluation of the relative importance of carotenoids to cutaneous bacteria in wild, captive and post-release frogs. It would also allow for the quantification of the overall effects of captivity on *P. corroboree* cutaneous bacteria, and would assist in characterising common communities of cutaneous bacteria. Wild *P. corroboree* were not available for this study due to there being very few animals left in the wild, but opportunities might present themselves in the future if reintroduction programs are

successful. Further comparison with other captive populations of *P. corroboree* would also be beneficial to further our understanding of the influence of different captive conditions on cutaneous bacteria communities, and frog immunity.

As expected, a comparison of bacteria species found across different amphibian species demonstrates significant differences in community composition (Antwis et al., 2014; Kueneman et al., 2014; Michaels et al., 2014; Shaw et al., 2014). However, similar groupings were found at family level, with grouping particularly evident in four families (*Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae* and *Xanthomonadaceae*) (Antwis et al., 2014; Kueneman et al., 2014; Michaels et al., 2014; Shaw et al., 2014)*. Within the present study, three bacterial species (*Escherichia coli*, *Acinetobacter calcoaceticus* and Unidentified C) were isolated from carotenoid-supplemented frogs, but not un-supplemented frogs. *Pseudomonas luteola* was also more commonly identified on carotenoid-supplemented frogs and contributed most to dietary treatment dissimilarity. Similarly, Antwis et al. (2014) isolated two bacteria species (*Staphylococcus sp*. and *Stenotrophomonas sp*.) from supplemented frogs, but not un-supplemented frogs. Taken together, these findings suggest that carotenoids may be particularly influential on specific bacterial species and may allow for growth where it would otherwise not be possible. By comparison, only one bacteria species (Unidentified E) was found on un-supplemented individuals but not supplemented individuals, suggesting that carotenoids may negatively impact the growth or survival of this species.

However, these treatment-specific species occurred in low numbers of individuals, suggesting that their presence or absence did not have a major impact on the overall outcomes of this study. Nevertheless, future work would benefit from further investigating species-specific effects of carotenoids on bacteria, particularly if these species were found to be substantially beneficial to amphibian immunity (such as the ability of *J. lividum* to inhibit growth of *B. dendrobatidis*) (Becker et al., 2009). As emphasised by these comparisons, the cutaneous bacteria associated with amphibians differ vastly between species and across geographic locations (Antwis et al., 2014; Kueneman et al., 2014; Michaels et al., 2014; Shaw et al., 2014). Further evidence of spatial and temporal taxonomic diversity was provided in a recent study which concluded that amphibian cutaneous bacteria differs across species, space and life history stages (Kueneman et al.,

2014). These differences suggest that there may not be a broad spectrum solution when relying on amphibian cutaneous bacteria to inhibit pathogen infection. However, in the case of *P. corroboree*, a small habitat range, and reduced genetic diversity, may allow for species-wide solutions.

Despite a lack of species-specific literature, bacteria species are known to have a variety of interactions with host species, including mutualistic, commensal and pathogenic relationships (Bletz et al., 2013; Cramp et al., 2014). Specific bacteria-host interactions in this study are not currently known. However in this particular case, there have been no previous signs of infection or stress within the captive *P. corroboree* population used in this study, suggesting that bacteria species present have a mutualistic/commensal relationship on the host amphibian, at least in their current abundance and relative proportions. Although it is important to note that some bacteria species may be opportunistic, thus tending towards pathogenic interactions following certain perturbations or density changes (Cramp et al., 2014). Developing an understanding of species-specific interactions between bacteria and *P. corroboree* is critical to understanding specific bacterial interaction mechanisms which may influence host immunity. For example, identifying the presence of specific bacteria species capable of antifungal metabolite production could be relevant to improving immunity within amphibian species such as *P. corroboree* (Becker et al., 2010; Becker et al., 2015; Brucker, Baylor, et al., 2008; Lam et al., 2010; Woodhams et al., 2007). Therefore, future work performing in-vitro inhibition assays of bacteria and pathogens (e.g. *B.dendrobatidis*) may allow for a targeted enhancement of bacteria species that are known to be beneficial to immunity in *P. corroboree*. This could be further enhanced by investigating the composition of bacterial communities in the natural habitat of *P. corroboree* to assess whether certain species are useful for enhancing host immunity. Given the limited availability of bacteria within captive husbandry environments, bacteria present in the colony may in part originate from live crickets that are bought into the captive facility to feed the frogs. Assuming this is the case, it could be argued that populations of beneficial bacteria could also be supplemented through the intentional introduction of target species. This could be achieved, for instance, by using substrates such as sphagnum moss that are sourced from the frog's natural range.

It is also important to note that relative abundances of bacteria species can affect species interactions between bacteria and their host. Relative abundances within the bacteria community were not quantified in this study, but gathering this data in future studies could improve our understanding of the prevalence and effects of particular bacteria species on the skin of captive *P. corroboree*. This would also aid in the diagnosis of pathogenic microbial infections in captive and wild individuals. Relative abundance data would also allow us to better understand competition mechanisms operating within cutaneous communities, which potentially result in the dominance of particular species. This may be a potential cause for lower species richness within un-supplemented individuals. It is also important to recognise that bacteria culturing methods used in the present study are likely to have only captured a fraction of the existing cutaneous community of *P. corroboree,* and thus may not be wholly representative of the bacterial community characterising this frog (Kong, 2011). Further investigation using a variety of culture techniques, aided by contemporary identification technology such as MALDI-TOF, may allow for a more comprehensive assessment of the cutaneous community.

Despite the results of the present study being in the predicted direction, it is necessary to consider the possibility that the findings might be dose specific. Experimental individuals received a carotenoid dose of 20 mg/g as tadpoles, but it is currently unknown whether this dosage is biologically relevant to *P. corroboree.* This dose was based on a previous study which determined that 20 mg/g dietary carotenoids significantly enhanced survival, development and fitness determining traits in *Xenopus tropicalis* (Ogilvy & Preziosi, 2012). Post-metamorphic carotenoid dose is not explicitly known due to discrepancies in fly and cricket consumption by frogs. However, a dose of 0.25 mg/g to fruit flies was based on a previous study which determined that a similar dose of dietary carotenoids significantly enhanced female growth, reproductive success and colour in *A. callidryas* (Ogilvy et al., 2012a, Ogilvy et al., 2012b). Additionally, carotenoid dose has been shown to have positive effects at intermediate levels (Arnold et al., 2010), but potential negative (pro-oxidant) effects at high concentrations (Larcombe et al., 2008; Vinkler & Albrecht, 2010). Therefore it is important to consider that different doses may have different effects on organismal health. However, Antwis et al. (2014) administered a carotenoid dosage of 5.0mg/g to *A. callidryas* that was twenty times higher than Ogilvy et al. (2012b) and four times lower than

the present study (and Ogilvy & Preziosi, 2012). This dose was also shown to significantly enhance the bacteria community of amphibian skin, as was the dose in the present study. Past research on the same captive *P. corroboree* population has indicated that the present dose significantly improves skin colouration but has limited effects on foraging performance, survival, growth rate, body size and condition of *P. corroboree* (Bailey, 2014; McInerney, 2014). Taken together, the above outcomes suggest that carotenoid supplementation in amphibians may have positive effects over a relatively broad range of dosages. Nevertheless, future work would benefit from establishing dose-response relationships to allow for the optimisation of carotenoid supplementation in captivity. Future studies would also benefit from gaining an understanding of natural carotenoid levels received by *P. corroboree,* which would allow for ecologically relevant carotenoid supplementation in captivity.

In summary, this study provides empirical evidence to support the hypothesis that dietary carotenoid supplementation enhances the cutaneous bacteria community of *P. corroboree*. This is only the second study examining these associations in amphibians and therefore would be complemented by further investigation into the influence of captive conditions on immune function in *P. corroboree* and other threatened amphibians. As mentioned previously, the present study was based on the assumption that an optimal bacteria community is one with high levels of diversity and abundance (Longo et al., 2015). Despite being well supported, this theory is yet to be investigated thoroughly in amphibian cutaneous communities. Additionally, *P. corroboree* is considered a critically endangered species by the IUCN, and thus, individuals available for this study were limited to our experimental population. However, present research outcomes justify further research incorporating other captive and wild populations to better understand dietary carotenoid mechanisms operating within *P. corroboree* cutaneous bacteria communities. Despite these limitations, this study provides a substantial building block for further investigation into optimal *ex-situ* conditions for maximising the mutualistic properties of cutaneous bacterial communities. This is critical because these captive conditions can have a direct effect on the immune capabilities of *P. corroboree* within captivity, and may also significantly influence immune function and survivorship post-release.

5. Conclusion

This study characterised the cutaneous bacterial community of the critically endangered Southern Corroboree Frog (*Pseudophryne corroboree*) in an *ex-situ* context and tested the effect of dietary carotenoid supplementation on these cutaneous bacterial populations. Dietary carotenoid availability was manipulated throughout juvenile and post-metamorphic life stages to determine whether dietary carotenoid supplementation enhanced abundance, species richness and affected overall community composition of cutaneous bacterial communities. The provision of dietary carotenoid significantly increased abundance and species richness and affected overall community composition of bacteria associated with the skin of *P. corroboree*. These findings provide support for the hypothesis that dietary carotenoid supplementation can enhance the cutaneous bacteria community of amphibians. The present study provides evidence to suggest that captive conditions such as diet can potentially influence the immune function of amphibians in captivity and thus may also impact on post-release health.

These outcomes are likely to be a result of bacterial-mucous interactions on the skin of the amphibian host promoting the growth, survival and reproductive capabilities of cutaneous bacteria. Additionally, the results obtained in the present study may be a product of carotenoid-enhanced survival of bacteria upon ingestion by the host as a result of amphibian sloughing processes. These explanations are based on the assumption that an optimal bacteria community is one with high levels of diversity and abundance, but this is yet to be confirmed for amphibian cutaneous communities. Placed in a broad context, this is only the second empirical study to examine the effects of dietary carotenoid supplementation on amphibian cutaneous bacteria. Consequently, the findings of the present study have helped lay the foundations for further investigation into the influence of captive conditions on amphibian cutaneous bacteria. Outcomes of the present study build upon a growing body of knowledge regarding the development of standardised *ex-situ* breeding conditions for endangered amphibians. These conditions are intended to maximise the mutualistic properties of cutaneous bacterial communities, thus improving the immune capabilities of amphibians both within captivity and upon release, and potentially leading to the suppression of amphibian pathogens such as *B.dendrobatidis.*

6. References

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7. Appendices

7.1. Appendix 1: Determination of appropriate dilution for bacterial suspension

i) Aim

A preliminary study was conducted to determine the optimum bacterial suspension dilution for growth and accurate quantification of *P. corroboree* cutaneous bacterial community.

ii) Methods

Sample Collection

Captive bred frogs were selected from a captive population within the University of Wollongong, Australia in April 2015. Frogs were selected from two dietary treatment groups: treatment 1 receiving carotenoid supplementation as juveniles and fed on a basal diet with no dietary carotenoid supplementation as adults (n = 12); and treatment 2 receiving a basal diet with no dietary carotenoid supplementation as juveniles and dietary carotenoid supplementation as adults (n = 12). Due to limited individuals available from the captive population for the main experiment, the treatment groups used in pilot experiments differ to those of the main experiment. However, given the preliminary purpose of this pilot experimentation, these additional treatment groups were a sufficient proxy for main experiment treatment groups.

Frogs were removed from individual housing containers) rinsed once with 30mL R.O. (Reverse Osmosis) water to remove transient bacteria, ensuring minimal handling to prevent loss of cutaneous bacteria. Individuals were handled with separate sterile gloves (Skinshield powder free latex gloves, Livingstone International, Australia) and contacted surfaces were sterile to prevent cross contamination of cutaneous bacteria (Antwis et al., 2014; Lauer, Simon, Banning, Lam, et al., 2007).

Cutaneous bacteria samples were collected using sterile collection swabs (Livingstone International Pty Ltd, Australia) using a swabbing procedure adapted from Shaw et al., 2014, Flechas et al., 2012 and Antwis et.al 2014. Frogs were swabbed (1swab/frog) following the same protocol as described in section 2.5. Swabs were placed in the designated storage tube and individual frog details were recorded (Frog ID number, Dietary treatment and Date of sample collection). Swabs were sealed in zip lock bags and stored at 20°C within a small esky while being transferred to the lab for processing (storage time = 10 hours). Particular care was taken to ensure the frogs were not harmed throughout the process and individuals were monitored post-swabbing for 4 weeks to ensure there were no signs of distress/injury as a result of the swabbing procedure.

Bacterial Culture

Using aseptic technique within a biosafety cabinet (Biological Safety Cabinet Class II, Email Westinghouse Pty Ltd, Australia), each swab was removed from the transport media and the swab tip cut off into a 1.5mL sterile Eppendorf tube (Eppendorf, Germany) containing 1000μL 0.9% sterile NaCl (Antwis et al., 2014; Nalven, 2013). Tubes containing swab tips were vortexed for 30 seconds to disassociate bacteria from the tip (Antwis et al., 2014). Swab tips were then removed from the tubes using sterile tweezers and solutions were vortexed for a further 5 seconds to ensure a homogenous bacterial suspension prior to serial dilution and plating. A serial dilution was performed to produce 4 bacterial suspensions per swab: 10^1 undiluted solution; 10^{-1}; 10^{-10}; 10^{-100} (total suspension volume = 1000μL). All solutions were vortexed for a further 5 seconds immediately before plating.

Each dilution per swab was plated by transferring 100μL aliquot of bacterial suspension to an LB (Luria Broth) nutrient agar media plate (100mm diameter) (AMRESCO®, USA) and spread evenly over the surface using a sterile plate spreader. One randomised additional plate was prepared per frog. Plates were allowed to dry for 10 minutes within hood prior to being inverted and sealed with parafilm® (Bemis Company, Inc., USA). Plates were incubated at 20°C (as determined by a pilot study-Appendix 2) and checked every 24±1 hours. Photographs were taken once growth had occurred with the aid of a backlight, including scale and appropriate label (frog ID, diet treatment, incubation time, incubation temperature and date). Photographs were analysed using ImageJ (National Institute of

Health, USA)) to obtain total abundance counts of CFU's (Colony Forming Units). Monitoring of growth was concluded once CFU numbers were consistent over three consecutive days, growth became too extensive or fungi obscured bacteria colonies.

Data analysis

Average bacterial abundance for each serial dilution was quantified and compared against all dilutions using an Analysis of Variance (ANOVA) model. Post-hoc Tukey's HSD analysis were used to determine differences between treatments. Prior to running the model, the assumptions of analysis were tested using Shapiro-Wilk's normality test and the Levene's test for homoscedasticity. No data transformations were required. Optimal bacterial growth was selected on the basis of total CFU range within 25-300 CFU (Sutton, 2011). Statistical analysis to determine optimal dilution was not possible.

iii) Results

Bacterial abundance was significantly different between dilution factors (ANOVA: $F_{3,20}$ = 12.6255, p = 0.0001). The 10^1 undiluted solution ranged from 23 CFU/plate to 280 CFU/plate (MEAN ± SEM = 142.17±37.50). The 10^{-1} dilution ranged from 2 CFU/plate to 47 CFU/plate (MEAN ± SEM = 24.167±7.10). The 10^{-2} and 10^{-3} dilutions had negligible bacterial growth (MEAN ± SEM = 2±0.86, 0.167±0.167). Accurate CFU counts are achieved where 25-300 CFU grow per plate (Sutton, 2011), average CFU counts were within this range at dilutions of 10^1 and 10^{-1} (Figure 7).
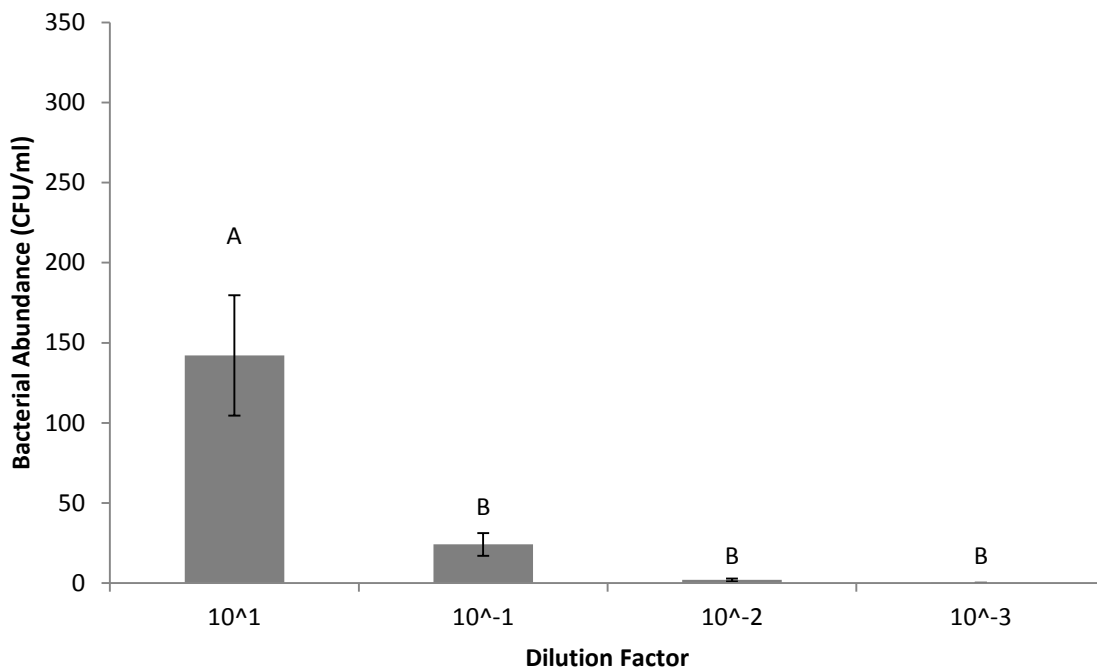
Figure 7. Average bacterial abundance (colony forming units (CFU) per mL) ± SEM isolated from the skin of *P. corroboree* across four dilution factors. CFU counts are considered sufficiently accurate between 25-300 units (Sutton, 2011). CFU/mL is equivalent to total culturable CFU obtained from sampling of each individual. Letters (A, B) represent outcome of a post-hoc Tukey's HSD test.

iv) Conclusions

Optimal bacterial growth occurred on both the 10^1 and 10^{-1} dilution bacterial solutions. Given that bacterial CFU is highly variable and accurate counts can only be obtained within a specific range (25-300 CFU), each main experiment frog swab should be plated in replicate using both of these solutions. This will ensure that variability in bacterial growth will not prevent this study from obtaining accurate and viable CFU counts.

7.2. Appendix 2: Determination of incubation temperature and time

i) Aim

A preliminary study was conducted to determine the optimum incubation temperature for growth of *P. corroboree* cutaneous bacterial community. This study also provides preliminary information to determine the most appropriate incubation time for the cutaneous bacterial community.

ii) Methods

Sample Collection

Captive bred frogs were selected from a captive population within the University of Wollongong, Australia in April 2015. Frogs were selected from 2 dietary treatment groups: treatment 1 receiving carotenoid supplementation as juveniles and fed on a basal diet with no dietary carotenoid supplementation as adults (n = 12); and treatment 2 receiving a basal diet with no dietary carotenoid supplementation as juveniles and dietary carotenoid supplementation as adults (n = 12). Due to limited individuals available from the captive population for the main experiment, the treatment groups used in pilot experiments differ to those of the main experiment. However, given the preliminary purpose of this pilot experimentation, these additional treatment groups were a sufficient proxy for main experiment treatment groups.

Frogs were removed from individual housing containers and rinsed once with 30mL R.O. (Reverse Osmosis) water to remove transient bacteria, ensuring minimal handling to prevent loss of cutaneous bacteria (Kueneman et al., 2014; Lauer et al., 2007; Shaw et al., 2014). Individuals were handled with separate sterile gloves (Skinshield powder free latex gloves, Livingstone International, Australia) and contacted surfaces were sterile to prevent cross contamination of cutaneous bacteria (Antwis et al., 2014; Lauer et al., 2007).

Cutaneous bacteria samples were collected via sterile collection swabs (Livingstone International Pty Ltd, Australia) using a swabbing procedure adapted from Shaw et al., 2014,

Flechas et al., 2012 and Antwis et.al 2014. Frogs were swabbed (1swab/frog) following the same protocol as described in section 2.5.1. Swabs were placed in the designated storage tube and individual frog details were recorded (Frog ID number, Dietary treatment and Date of sample collection). Swabs were sealed in zip lock bags and stored at 20°C within a small esky while being transferred to the lab for processing (storage time = 10 hours). Particular care was taken to ensure the frogs were not harmed throughout the process and individuals were monitored post-swabbing for four weeks to ensure there were no signs of distress/injury as a result of the swabbing procedure.

Bacterial Culture

Each swab was plated separately on LB (Luria Broth) nutrient agar media plate (100mm diameter) (AMRESCO®, USA) by streaking the swab directly across the agar surface 10 times in a broad zig-zag pattern. Plates were then inverted and sealed with parafilm® (Bemis Company, Inc., USA) prior to being randomly assigned to one of four temperature treatments 5°C, 10°C, 20°C and 30°C. Three frogs from each dietary treatment were assigned to each temperature category. Plates were checked every 24±1 hours and photographs were taken once growth had occurred with the aid of a backlight, including scale and appropriate label (frog ID, diet treatment, incubation time, incubation temperature and date). Every 24 hours, total bacterial abundance (CFU/streak) and bacterial species richness was quantified. Monitoring of growth was concluded once CFU numbers were consistent over three consecutive days, growth became too extensive or fungi obscured bacteria colonies. Peak bacterial growth was recorded and used for subsequent comparative analysis (occurred on various days for different temperature treatments–refer to figure 10). Optimum incubation temperature was selected based on a combination of three factors:

1) High bacterial abundance

2) High bacterial diversity (species richness)

3) Time efficiency of bacterial growth

Data Analysis

At optimal bacterial growth, average bacterial abundance (per swab) for each temperature treatment was quantified and compared against each temperature treatment using an Analysis of Variance (ANOVA) model. Average bacterial species richness for each temperature treatment was quantified and comparison amongst groups was also done using an ANOVA model. Post-hoc Tukey's HSD analyses were used to determine differences between treatments. Prior to running the models, the assumptions of analysis were tested using Shapiro-Wilk's normality test and the Levene's test for homoscedasticity. No data transformations were required. Differences between groups were statistically analysed (as above) but selection of optimal temperature treatment was performed on the basis of highest abundance and diversity measurements.

Average bacterial growth for each temperature treatment was quantified but this data could not be statistically assessed. However, this data was used for a direct comparison of average bacterial growth for each temperature treatment as a function of time so as to allow for the selection of an incubation temperature which promoted maximum growth in a time efficient manner.

iii) Results

Total cutaneous bacterial abundance differed between incubation temperature treatments (ANOVA: $F_{3,20}$ = 3.9804, p = 0.0225; figure 8). The $10^{o}C$ and $20^{o}C$ temperature treatments promoted significantly more bacterial growth (CFU/streak) (MEAN ±SEM = 118.33±34.96, 90.83±28.51, respectively) than the $5^{o}C$ temperature treatment (MEAN ± SEM = 44.83±21.35). A lack of bacterial CFU growth was observed in the $30^{o}C$ temperature treatment due to an overgrowth of fungi-like species and biofilm species.

Figure 8. The effect of incubation temperature (T = 5, 10, 20 & 30 °C) on bacterial abundance (Colony forming units (CFU) per streak) ± SEM of the culturable cutaneous bacterial community of *P. corroboree* (n = 6 frogs per treatment) at 72 hours. Letters (A, B, C) represent outcome of post-hoc Tukey's HSD test. CFU per streak is equivalent to CFU obtained from each individual.

Bacterial species richness differed between incubation temperature treatments (ANOVA: $F_{3,20}$ = 10.7011, p = 0.0002; figure 9). The 5°C, 10°C and 20°C temperature treatments supported a higher species diversity (MEAN ±SEM = 2.33±0.49, 2.33±0.61 and 3.5±0.43 respectively) than the 30°C (MEAN ±SEM = 1.33±0.21). A lack of bacterial growth was observed in the 30°C temperature treatment due to an overgrowth of fungi-like species and biofilm species. Average bacterial species richness was highest at 20°C (MEAN ± SEM = 3.50 ± 0.43), above all other incubation temperature treatments (5, 10 & 30°C), however this was only statistically significant compared to the 30°C temperature treatment (Tukey's HSD, p<0.05; Figure 9). There were no significant differences between the three lowest incubation temps (5, 10 & 30°C), but species richness in all of these treatment s was higher than at 30°C.
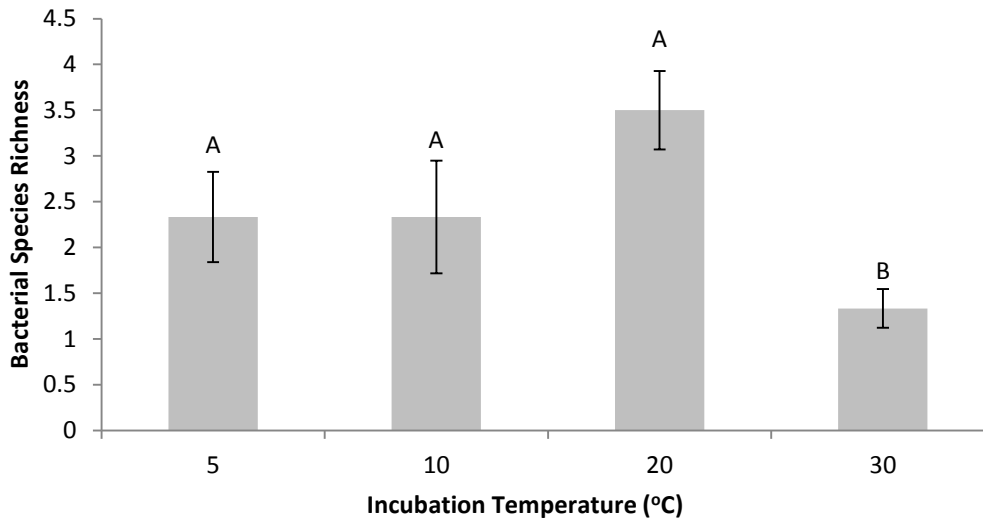
Figure 9. The effect of incubation temperature (T = 5, 10, 20 &30°C) on bacterial species richness ± SEM of the culturable cutaneous bacterial community of *P. corroboree* (n = 6 per treatment). Letters (A, B, C) represent outcome of post-hoc Tukey's HSD test.

Across the four temperature treatments, time until initial bacterial growth ranged from 24-168 hours (figure 10). Time until initial growth was inversely proportional to incubation temperature. Within the 5°C incubation temperature treatment, initial growth was present at 168 hours and stable CFU counts were obtained at 216hrs (figure 10a). Within the 10°C incubation temperature treatment, initial growth was present at 96 hours and stable CFU counts were obtained by 144 hours (figure 10b). At an incubation temperature of 20°C, initial growth was present at 48 hours and stable CFU counts were obtained by 144 hours (figure 10c). While in the 30°C temperature treatment, overgrowth of fungi and biofilm species were present at 24 hours (figure 10d).
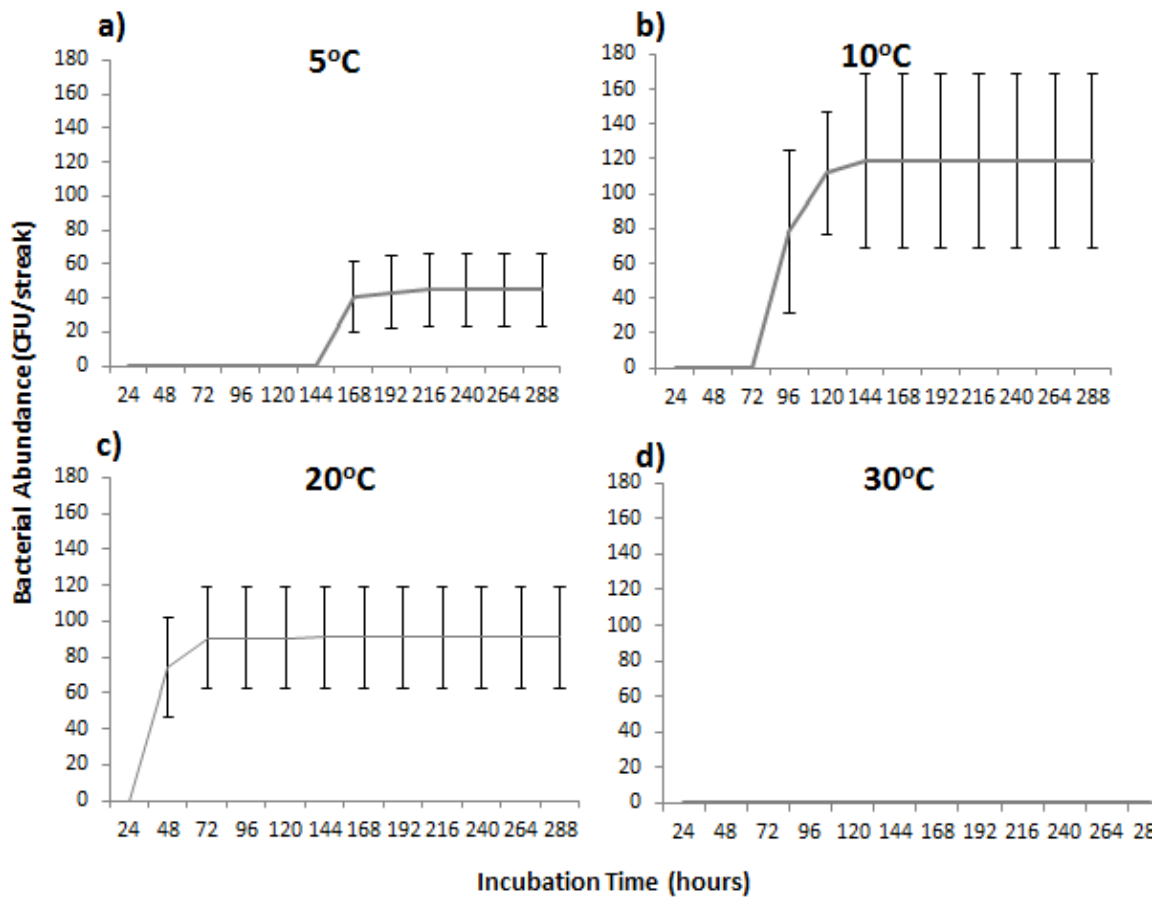
Figure 10. Cutaneous bacterial growth (colony forming units (CFU) per swab) ±SEM over time obtained from the skin of *P. corroboree* frogs across four incubation temperature treatments (a = 5°C, b = 10°C, c = 20°C and d = 30°C) (n = 6 frogs per treatment). CFU per streak is equivalent to CFU obtained from each individual.

iv) Conclusion

The conclusions of this study involve the best combination of three interacting factors:

1) High bacterial abundance: both the 10°C and 20°C incubation temperature treatments showed high levels of bacterial growth.

2) High bacterial species richness: The 5°C, 10°C and 20°C incubation temperature treatments showed significantly higher levels of bacterial species richness, with the 20°C having the highest average species richness.

3) The 20°C incubation temperature treatment demonstrated best time efficiency with consistent growth occurring at 72 hours onwards.

Therefore, the 20°C was selected as the optimal incubation temperature for most representative growth of *P. corroboree* cutaneous bacterial community. This was further confirmed as the appropriate temperature given that the frogs were also maintained at 20°C. As such, 72 hours was selected as the most appropriate incubation time to obtain accurate quantification of bacterial growth.

7.4. Appendix 3: Unidentified bacteria species descriptions

Unidentified A:

Small gram-positive rod, yellow colour, raised/convex colonies with entire margins, catalase positive

Unidentified B:

Small gram-positive rod, cream colour, convex/raised colonies with entire margin, catalase positive

Unidentified C:

Gram negative-rod, cream colour, glossy appearance, oxidase positive

Unidentified D:

Small gram-positive rod, cream colour, convex colonies with entire margins, catalase positive

Unidentified E:

Gram-negative rod, yellow/ cream colour, convex colonies with entire margins, oxidase positive

7.3. Appendix 4: Bacteria species assemblages of individual frogs

Table 2. Cutaneous bacteria species assemblages of carotenoid-supplemented (n =22) and un-supplemented (n = 22) *P. corroboree* frogs

Cutaneous Bacteria Species	Carotenoid-Supplemented Frogs (ID number below)																						Un-supplemented Frogs (ID number below)																					
	95	23	167	59	108	126	107	119	131	102	137	179	19	867	47	140	90	40	60	168	18	84	145	312	129	98	12	213	15	63	35	59	531	89	84	6	76	61	12	915	127	35	50
Acinetobacter calcoaceticus																	x																										
Aeromona hydrophila			x	x											x	x							x		x				x		x				x		x		x				x
Citrobacter braakii	x		x		x			x		x	x		x					x		x			x	x	x	x				x		x	x				x	x		x	x		x
Citrobacter freundii																x			x				x					x			x										x		
Escherichia coli								x																																			
Ewingella americana	x	x			x				x		x				x			x	x		x														x				x	x			
Klebsiella oxytoca					x					x	x	x					x			x	x	x				x			x									x	x	x		x	
Klebsiella pneumoniae sp		x		x							x		x																					x									
Morganella morganii	x	x	x	x			x	x	x		x		x			x	x	x	x	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Pasteurella pneumotropica					x					x																		x															
Proteus mirabilis	x	x			x	x		x	x	x	x		x	x					x	x	x				x	x		x		x		x		x		x		x		x	x	x	x
Pseudomonas luteola	x	x	x	x	x	x	x			x	x			x		x		x	x	x		x	x		x	x														x	x	x	

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
Pseudomonas oryzihabitans			x																																			x		x						
Rhodococcus erythopolis	x		x		x		x						x		x	x	x	x			x													x	x		x				x					
Serratia marcescens														x										x																			x			
Serratia sp.	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x		x		x	x			x	x				x	x		x	x			x	x		x	x
Stenotrophomonas maltophilia	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Variovorax paradoxus	x		x	x		x	x	x		x		x			x	x	x		x		x		x	x		x		x	x	x	x		x								x					x
Unidentified A	x		x	x		x	x		x	x		x		x		x	x		x							x	x		x		x	x				x										x
Unidentified B	x	x		x		x	x		x	x	x	x	x	x			x	x		x		x		x	x	x	x	x		x	x	x			x	x			x	x						
Unidentified C	x		x	x	x			x	x					x			x																													
Unidentified D												x									x	x	x										x		x											x
Unidentified E																															x			x		x	x		x		x					x