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The influence of carotenoid supplementation at different life-stages on the foraging performance of the Southern Corroboree frog (*Pseudophryne corroboree*): a test of the Silver Spoon and Environmental Matching Hypotheses

Emma McNerney

University of Wollongong, epm509@uowmail.edu.au

Aimee J. Silla

University of Wollongong, asilla@uow.edu.au

Phillip G. Byrne

University of Wollongong, pbyrne@uow.edu.au

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McNerney, Emma; Silla, Aimee J.; and Byrne, Phillip G., "The influence of carotenoid supplementation at different life-stages on the foraging performance of the Southern Corroboree frog (*Pseudophryne corroboree*): a test of the Silver Spoon and Environmental Matching Hypotheses" (2016). *Faculty of Science, Medicine and Health - Papers: part A*. 3604.
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Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

McInerney, E. P., Silla, A. J. & Byrne, P. G. (2016). The influence of carotenoid supplementation at different life-stages on the foraging performance of the Southern Corroboree frog (*Pseudophryne corroboree*): a test of the Silver Spoon and Environmental Matching Hypotheses. *Behavioural Processes*, 125 26-33.

The influence of carotenoid supplementation at different life-stages on the foraging performance of the Southern Corroboree Frog (*Pseudophryne corroboree*): a test of the Silver Spoon and Environmental Matching Hypotheses

Emma P. McInerney*, Aimee J. Silla, Phillip G. Byrne¹

*Corresponding author: epm509@uowmail.edu.au

Ph: +61 2 4298 1932

¹Centre for Sustainable Ecosystem Solutions, School of Biological Sciences, University of Wollongong, Wollongong, New South Wales, 2522, Australia.

Word Count= 5271

Figures = 3

Tables = 0

Abstract

Organismal performance can be significantly affected by the nutritional conditions experienced during different life-stages. The Silver Spoon Hypothesis predicts that individuals will always perform better as adults when they experience advantageous conditions during development. In contrast, the Environmental Matching Hypothesis predicts that individuals will perform better if they experience similar conditions during development and adulthood. Past tests of these hypotheses have focussed on the effect of food quantity on growth and development, with little attempt to investigate the effect of individual nutrients on behavioural traits. This study aimed to test the predictions of the Environmental Matching and Silver Spoon Hypotheses by investigating the influence of carotenoid supplementation at different life-stages on the foraging performance of *Pseudophryne corroboree*. To assess foraging performance, adults were presented with prey in either a cryptic or conspicuous foraging matrix. There was no effect of diet treatment on time to first movement towards prey, number of stalking events, time spent actively foraging, proportion of successful strikes, proportion of prey consumed or number of pedal luring events. These findings indicate that carotenoid supplementation at different life-stages does not influence the foraging performance of *P. corroboree*, providing no support for either the Silver Spoon or Environmental Matching Hypotheses.

Keywords; antioxidant; anuran; behaviour; diet; development; nutrition

1. Introduction

Variation in the environmental conditions experienced during developmental life-stages can shape adult phenotype and performance (Monaghan, 2008; Butler and McGraw, 2012; Hopwood et al., 2014). Diet and nutrition are two environmental factors known to have a direct effect on morphological, physiological and behavioural traits which determine performance (e.g. Møller, et al., 2000; Svensson and Wong, 2011). However, what remains poorly understood is how diet and nutrition received at different life-stages (i.e. developmental versus adult life-stages) interact to influence individual adult performance (Møller et al., 2000; Monaghan, 2008; Dmitriew and Rowe, 2011; Butler and McGraw, 2012; Hopwood et al., 2014).

At present, there are two main hypotheses that consider the influence of environmental conditions experienced during different life-stages on adult performance and fitness (reviewed by Monaghan, 2008). First, the Environmental Matching Hypothesis predicts that individuals experiencing the same conditions in developmental and adult life-stages will have a fitness advantage over individuals experiencing mismatched conditions during developmental and adult life-stages (Monaghan, 2008). The proposed reason for this is that adult phenotypes should perform optimally in environments that match those in which the juvenile phenotype developed. Second, the Silver Spoon Hypothesis predicts that individuals will have higher fitness in the adult life-stage when they experience good conditions during the developmental life-stage, irrespective of conditions experienced during the adult life-stage (Monaghan, 2008). It is hypothesised that individuals experiencing good conditions in the developmental life-stage are able to invest more resources into fitness-determining traits, providing an advantage later in life (Monaghan, 2008).

To date, empirical tests of the Environmental Matching and Silver Spoon Hypotheses have primarily focused on investigating how the quantity of food received at different life-stages impacts fitness-determining traits (Dmitriew and Rowe, 2011; Hopwood et al., 2014). Interestingly, the majority of these studies have provided support for the Silver Spoon Hypothesis (see Ogilvy et al., 2012, Wong and Kölliker, 2014), with relatively few studies providing support for the Environmental Matching Hypothesis (but see Butler and McGraw, 2012). It is well established that an increase in the nutritional quality of food consumed can be just as beneficial to individuals as an increase in the quantity of food consumed (Møller et al., 2000). However, it is only in recent years that studies have begun to investigate how access to individual compounds at different life-stages influences organismal performance (Butler and McGraw, 2012; Ogilvy et al., 2012).

One group of compounds gaining an increasing amount of research attention are carotenoids: a group of over 600 compounds produced by plants, bacteria and fungi that are obtained by animals

through diet (Svensson and Wong, 2011). Carotenoids are known to aid in animal colouration, growth, immune response and vision (Landrum and Bone, 2001; Svensson and Wong, 2011; Ogilvy et al., 2012). Despite this knowledge, a thorough understanding of how variation in carotenoid availability at different life-stages influences adult phenotype and performance is currently lacking. Very few studies have investigated how carotenoid supplementation at different life-stages affects adult fitness-determining traits, and these studies have generated mixed support for the Environmental Matching and Silver Spoon Hypotheses (see Butler and McGraw, 2012; Ogilvy et al., 2012; Butler et al., 2013). Ogilvy (2012) manipulated dietary carotenoid intake in Red-Eyed Tree frogs (*Agalychnis callidryas*) and showed that individuals fed a diet high in carotenoids as juveniles had redder dorsums and toe pads in the adult life-stage compared to those reared on a control diet, providing support for the Silver Spoon Hypothesis. In contrast, Butler and McGraw (2012) manipulated carotenoid availability at different life-stages in Mallard ducks (*Anas platyrhynchos*) and found that individuals maintained on a carotenoid-supplemented diet in both juvenile and adult life-stages had brighter colouration, as predicted by the Environmental Matching Hypothesis. Furthermore, a second study in Mallard ducks investigated the influence of carotenoids on testis size and provided no support for either the Silver Spoon or Environmental Matching Hypotheses, showing instead that a carotenoid-rich adult diet led to reduced testis size (Butler et al., 2013). Taken together, the findings of these studies suggest that the influence of dietary carotenoids on adult phenotype may be species specific and/or dependant on the traits examined.

To determine the general relevance of the Environmental Matching and Silver Spoon Hypotheses, there is a need to investigate the influence of dietary conditions at different life-stages upon a variety of fitness-determining traits. In particular, behavioural traits require attention because they have been largely overlooked by past studies. Foraging behaviour is one behavioural trait that could be strongly impacted by carotenoid intake at different life-stages due to their positive effect on visual acuity, which is important for active predators that must locate and capture cryptic prey items (Bond and Kamil, 2002). Dietary carotenoid supplementation may affect vision by increasing the level of macular pigment in the eye, which is responsible for detailed vision (Landrum and Bone, 2001; Carpentier et al., 2009; Lien and Hammond, 2011). Additionally, the consumption of carotenoids can limit damage caused by oxygen radicals in the retina, which, are generated when blue and UV light enter the eye (Landrum and Bone, 2001; Carpentier et al., 2009; Lien and Hammond, 2011). These harmful oxygen radicals cause macular degeneration and a deterioration of visual acuity (Landrum and Bone, 2001; Carpentier et al., 2009; Lien and Hammond, 2011). Therefore, it is reasonable to predict that the consumption of carotenoids may improve visual acuity and, in turn, increase the probability of successful prey detection and capture.

While several studies have demonstrated beneficial effects of dietary carotenoids on foraging performance (Raguso et al., 2007; Goyret et al., 2009; Li et al., 2009; Toomey and McGraw, 2011), it

remains unknown whether foraging performance is affected by variation in the availability of carotenoids at different life-stages (Ragusso et al., 2007; Goyret et al., 2009; Li et al., 2009; Toomey and McGraw, 2011). Therefore, this is an area that requires empirical attention. One vertebrate group where such effects could be easily tested are anuran amphibians. Many anuran species are visual predators (Wells, 2007), and most of these species are characterised by a bi-phasic lifecycle with the process of metamorphosis providing an obvious separation between developmental and adult life-stages (Rollins-Smith, 1998). This separation makes it possible to reliably manipulate carotenoid availability at different life-stages. The aim of this study was to test the predictions of the Environmental Matching and Silver Spoon Hypotheses by investigating the influence of carotenoid supplementation at developmental and adult life-stages on the foraging performance of the Southern Corroboree frog (*Pseudophryne corroboree*).

2. Methods

2.1 Study species

P. corroboree is a small (25-30 mm snout vent length) toadlet (family: Myobatrachidae) endemic to Kosciuszko National Park in the Snowy Mountain region of New South Wales (Osborne, 1991). *P. corroboree* is a brightly coloured species with distinct yellow and black markings on the dorsal and ventral surfaces (Osborne, 1991). The diet of *P. corroboree* consists of algae and/or other organic matter during the developmental life-stage, and ants and other small insects in the adult life-stage (Osborne, 1991; Daly, 1998). Because these dietary items contain high levels of carotenoids (Lichtenthaler, 1987; Osborne, 1991; Eeva et al., 2010), it is assumed that carotenoids are present in the natural diet of *P. corroboree* during all life-stages.

2.2 Study animals

A total of 96 fertilised *P. corroboree* eggs were obtained from a captive colony maintained at Melbourne Zoo, Australia and transported to the Ecological Research Centre at the University of Wollongong, Australia on the 19th of July, 2013. Eggs were generated from matings between six males and twelve females, resulting in 15-28 unique sire and dam pairings, depending on whether females of this species are polyandrous and divide eggs between multiple male. Following arrival, eggs were stimulated to hatch by flooding with reverse-osmosis (R.O.) water. All tadpoles hatched within 11 days, and following hatching were immediately transferred to experimental aquaria (10.5 cm x 10 cm x 10 cm). Tadpoles were then reared to metamorphosis (defined by full tail absorption (Gosner stage 46)) and during the developmental (tadpole) and adult (post-metamorphic) life-stages individuals were exposed to different diet treatments (see Section 2.3).

2.3 Experimental design

To test the influence of dietary carotenoids received at different life-stages on the foraging performance of *P. corroboree*, individuals ($n = 24$ per treatment) were reared under four diet treatments: (1) individuals were reared on a carotenoid-supplemented diet during both the developmental (tadpole) and adult (post-metamorphic) life-stages (C-C), (2) individuals were reared on a carotenoid-supplemented diet during development, but an un-supplemented diet as adults (C-U), (3) individuals were reared on an un-supplemented diet during development, but a carotenoid-supplemented diet as adults (U-C) and (4) individuals were reared on an un-supplemented diet during both the developmental and adult life-stages (U-U) (Fig. 1). A breakdown of the composition of the experimental diets as well as a detailed carotenoid profile for each of the experimental treatments is provided by Silla *et al.*, (2016).

Fig. 1: Experimental design of diet treatments in juvenile and adult life-stages. The letter C denotes treatments where carotenoid supplementation was provided, and the letters U denote treatments where carotenoid supplementation was not provided.

2.4 Developmental husbandry and nutrition

Tadpoles were housed individually in plastic aquaria (10.5cm x 10cm x 10cm) containing 600ml of reverse osmosis water (R.O. water). Animals were kept in a controlled temperature room with an 11.5 hr/12.5 h day-night lighting cycle which included 15 min of twilight (low level lighting) at both dawn and dusk. Additionally, individuals were provided with one hour of UV-B light per day.

provided by a single bulb (Reptisun 10.0 36" bulb; Pet Pacific, Emu Plains, NSW, Australia) suspended approximately 20 cm above the aquaria. Each aquarium received a partial water change (~50% volume) via an automated irrigation system (Aqua Systems, Melbourne, VIC Australia) connected to an R.O. water system (Sartorius Stedim Biotech, Göttingen, Germany). Three water changes occurred each week. Additionally, once a week excrement and excess food were siphoned from each aquarium using a 30 ml plastic syringe connected to a 15 cm length of aquarium tubing (3 mm ID).

Tadpoles were assigned to one of two diet treatment groups; (i) a basal (un-supplemented) diet: consisting of ground fish flakes only or (ii) a carotenoid-supplemented diet: consisting of the basal diet plus a carotenoid supplement. The basal diet consisted of 2 g of ground fish flakes (75:25 mixture of Sera Flora/Sera Sans; SERA, Heinsberg, Germany) suspended in 20ml of R.O. water. The carotenoid diet consisted of 2 g of ground fish flakes (75:25 mixture of Sera Flora/Sera Sans) supplemented with 0.04 g of carotenoid powder (Superpig; Rapashy®, CA, USA). The food mixture was suspended in 20 ml of R.O. water homogenised and drawn into 10ml syringes to allow for even distribution of food among aquaria. Each 10 ml syringe was frozen at -20°C and thawed immediately before use. Tadpoles received two droplets of food (2 drops = 0.0585-0.0685 g wet mass, 0.015-0.018 g dry mass), three times per week for the first eight weeks, then four droplets (4 drops = 0.117-0.137g wet mass, 0.03-0.036g dry mass) three times a week until metamorphosis. All food was consumed by individuals between feeding days. Upon forelimb emergence (Gosner stage 42), the water level was reduced to approximately 150 ml of R.O. water and partially submerged sponge was provided to enable tadpoles to leave the water. As tadpoles do not feed during metamorphosis, pre-metamorphic individuals were not provided with food. Experimental treatment had no significant effect on tadpole growth and development, and no significant effect on tadpole body size (head length, tail length or total length) (Byrne and Silla, unpublished data).

2.5 Adult husbandry and nutrition

Once metamorphosis was complete (full tail absorption; Gosner stage 46), all frogs were fed a diet of un-supplemented 10 day old *Acheta domestica* crickets twice a week until one month after the final frog metamorphosed, at which point experimental diets were applied. In the post-metamorphic un-supplemented diet (C-U, U-U) frogs were fed a diet of *A. domestica* crickets gut-loaded for 48 h with green apple, twice weekly. In the post-metamorphic carotenoid-supplemented diet (C-C, U-C), crickets were gut-loaded for 48 hours with carrots and were dusted with 1.0 g of carotenoid powder (Superpig; Rapashy®, USA). Once a week, *A. domestica* crickets in both diet treatments were dusted with 0.2 g of calcium powder (Repti-cal; AristoPet, Australia) to prevent calcium deficiencies (Lannoo, 2008). All food was consumed by individuals between feeding days. Throughout post-metamorphic life, individuals were housed in experimental containers (10.5 cm x 10 cm x 10 cm)

consisting of a layer of aquarium pebbles, covered by a layer of sphagnum moss (*Sphagnum cristatum*; Brunnings, Australia). Additionally, containers were flushed with approximately 500 ml of R.O. water once a week to ensure the removal of excrement and food. Experimental treatment had no significant effect on the growth and development of frogs, and had no significant effect on frog body size (snout vent length) (Byrne and Silla, unpublished data).

2.6 Quantifying foraging performance

Foraging performance assays were conducted on the 12th of January, 2015, when individuals were 74 weeks of age post hatching. Trials were performed between 09.00 and 14.00 h, approximately 70-75 h after feeding, which ensured frogs were hungry and motivated to forage. The prey items used were 10 day old *A. domestica* crickets dusted with 0.2 g of calcium powder to standardise prey colour and to increase or decrease the level of background matching. To determine the impact of dietary carotenoids on foraging performance, adult *P. corroboree* were exposed to a behavioural assay where the colour of prey items was either matched or mismatched with the colour of the foraging background (Fig. 2). Individuals from all diet treatments were allowed to forage on one of these two foraging backgrounds; (i) an environment where the colour of the prey and the background were matched (referred to hereafter as cryptic background) or, (ii) an environment where the colour of the prey and the background were mismatched (referred to hereafter as conspicuous background). The foraging backgrounds were controlled using laminated photographs of sphagnum moss that were adhered to the inside of each container. Sphagnum moss photographs were taken using a Canon IXUS 240 HS camera (Canon, Japan) under the standardised lighting described above (see Section 2.4). Each background type was created by manipulating the colouration of a raw sphagnum moss photograph using the 'Replace Colour' tool in Photoshop CC (Adobe, USA). In order to increase the background matching of calcium dusted *A. domestica* crickets, a photograph of calcium dusted crickets was taken under standard lighting (see Section 2.4). The four most dominant colour values of the photograph were recorded and used to manipulate the sphagnum moss photograph, resulting in a cryptic background (Fig. 2a). Additionally, the colours of the cryptic background image were inverted in Photoshop CC, creating a conspicuous background (Fig. 2b). Only colour vision was tested in this experiment as previous studies have suggested that anurans are only visually sensitive to the visible proportion of the electromagnetic spectrum (Liebman and Entine, 1968; Partridge et al., 1992).

Fig. 2: Photographs of calcium dusted *A. domestic* crickets on (a) a prey colour matched foraging background (cryptic) and (b) a prey colour mismatched foraging background (conspicuous).

Foraging trials consisted of individuals being placed in plastic experimental containers (12 cm x 12 cm x 13 cm) with twenty prey items per container (10 day old *A. domestica* crickets). The number of crickets used was selected based on the results of a pilot study which indicated that the proportion of prey items consumed had a lower variance at a density of 20 crickets compared with three other densities (5, 10 or 15 crickets per container; $n = 6$ frogs per treatment, Levene's test, $F_{3,20} = 19.0979$, $P < 0.0001$). Foraging trials were run for a total of 20 min, including a 10 min acclimation period. Individuals used in the trials had been exposed to one of the four diet treatments (C-C, C-U, U-C or U-U). This created a total of eight treatments ($n = 8$ frogs per treatment); (1) C-C frogs with cryptic prey, (2) C-C frogs with conspicuous prey, (3) C-U frogs with cryptic prey, (4) C-U frogs with conspicuous prey, (5) U-C frogs with cryptic prey, (6) U-C frogs with conspicuous prey, (7) U-U frogs with cryptic prey and (8) U-U frogs with conspicuous prey. During the acclimation period, frogs were held under a plastic opaque cup (4 cm x 3.5 cm x 3.5 cm) to ensure they were unable to see or interact with prey. Each trial began when the cup was lifted and the frog was permitted to forage. Trials were video recorded using two cameras, a Sony Exmore R Handycam (Sony, Japan) and a Canon HD Legria camcorder (Canon, Japan) that simultaneously recorded eight trials ($n = 4$ trials per camera) positioned approximately 40 cm above containers. Six response variables were measured in each assay using EthoLog 2.2 behavioural software (Ottoni, 2000). These were: (1) time to first movement towards a prey item (sec), (2) number of stalking events, (3) proportion of time spent actively foraging (total time spent stalking/ total trial time after first movement towards a prey item), (4) proportion of successful strikes (number of prey items consumed/ number of strikes), (5) proportion of prey items consumed (number of prey items consumed/ total number of prey items presented) and (6) number of pedal luring events. Pedal luring is characterised by the rapid movement

of one or both of the outer digits on the rear feet, a behaviour thought to be used to lure prey in several anuran species, including *P. corroboree* (see Ref. McFadden et al., 2010).

2.7 Statistical analyses

To test the effect of diet treatment and foraging background on the number of stalking events and the proportion of time spent actively foraging, two separate Two-Factor Analysis of Variance (ANOVA) models were used. The explanatory variables for each ANOVA model were diet treatment and foraging background, and the response variable was either; number of stalking events or proportion of time spent actively foraging. Assumptions of normality and homoscedasticity were tested using Shapiro-Wilk's tests and Levene's tests respectively. All data met the assumption of homoscedasticity and normality, with the exception of proportion of time spent actively foraging, which was consequently arcsine transformed prior to analyses. Within each treatment, post-hoc treatment comparisons were made using Tukey's HSD tests. To test for the influence of diet treatment and foraging background on time to first movement towards a prey item, a Generalised Linear Model (GLM) with normal distribution and an identity link function was used. To test for the influence of diet treatment on number of pedal luring events a GLM was run using a Poisson distribution and a log link function. To test for the influence of diet treatment on proportion of successful strikes and proportion of prey items consumed, two separate GLMs were run. These models used a binomial distribution and a logit link function. In each of the GLM models, the explanatory variables were diet treatment and foraging background (including an interaction term between treatment and foraging background) and the response variable was either; time to first movement towards a prey item, proportion of prey items consumed, proportion of successful strikes or number of pedal luring events. The test statistics used for all GLMs were Maximum Likelihood Chi-squared tests. For each GLM model, post-hoc treatment comparisons were made using Wilcoxon Each Pair tests. Within each diet treatment, body size (snout vent length) did not have a significant influence on any of the response variables measured. As such, body size was not included as a covariate in any subsequent statistical analyses. All statistical analyses were conducted in the software package JMP Pro 11 (SAS, USA).

3. Results

3.1 Time to first movement towards a prey item

Across treatment groups, time to first movement towards a prey item ranged from 0.35 to 131.63 s. (mean $\pm$ SEM = 23.21 s $\pm$ 2.58; Fig. 3a). Time to first movement towards a prey item was not significantly affected by diet treatment (GLM, $\chi^2_3 = 1.4567053$, $P = 0.6923$) and there was no significant interaction between diet treatment and foraging background (GLM, $\chi^2_3 = 4.0815984$, $P = 0.2528$). Frogs on conspicuous backgrounds did, however, take significantly longer to move towards

their first prey item than those on cryptic backgrounds (mean $\pm$ SEM, cryptic background = 18.16 ± 2.63 , mean $\pm$ SEM, conspicuous background = 28.26 ± 4.30 ; GLM, $\chi^2_1 = 4.354492$, $P = 0.0369$).

Fig. 3:Effect of diet treatment on (a) time to first movement towards a prey item,(b) number of stalking events, (c) proportion of time spent actively foraging, (d) proportion of successful strikes, (e) proportion of prey items consumed and (f) number of pedal luring events ($n = 8$ frogs per treatment). Data shown are untransformed means $\pm$ SEM. Letters represent the results of post-hoc Tukey's tests and Wilcoxon Each Pair tests. Treatments that share a letter are not significantly different from one another. The four diet treatments were; (i) individual reared on a carotenoid-supplemented diet during both the developmental and adult life-stages (C-C), (ii) individuals reared on a carotenoid-supplemented diet during development, but on an un-supplemented diet as adults (C-U), (iii) individuals reared on an un-supplemented diet during development, but on a carotenoid-supplemented diet as adults (U-C) and (iv) individuals reared on an un-supplemented diet in both the developmental and adult life-stages (U-U).

3.2 Number of stalking events

Across treatment groups, the number of stalking events ranged from 0 to 15 (mean $\pm$ SEM = 5.07 ± 0.25 ; Fig. 3b). There was no significant effect of diet treatment on number of stalking events (Two-Factor ANOVA, $F_{3,3} = 0.2879$, $P = 0.8339$). Similarly, there was no influence of foraging background on the number of stalking events (mean $\pm$ SEM, cryptic background = 6.91 ± 0.55 ; mean $\pm$ SEM, conspicuous background = 5.72 ± 0.54 ; Two-Factor ANOVA, $F_{1,3} = 2.2274$, $P = 0.1412$), and there was no interaction between diet treatment and foraging background (Two-Factor ANOVA, $F_{3,3} = 0.4340$, $P = 0.7295$).

3.3 Proportion of time spent actively foraging

Across treatment groups, the proportion of time frogs spent actively foraging ranged from 0 to 0.70 (mean $\pm$ SEM = 0.21 ± 0.01 ; Fig. 3c). There was no significant effect of diet treatment on the proportion of time spent actively foraging (Two-Factor ANOVA, $F_{3,3} = 0.6579$, $P = 0.5814$). Additionally, there was no influence of foraging background on the proportion of time frogs spent actively foraging (mean $\pm$ SEM, cryptic background = 0.18 ± 0.02 ; mean $\pm$ SEM, conspicuous background = 0.21 ± 0.02 ; Two-Factor ANOVA, $F_{1,3} = 0.7733$, $P = 0.3830$) and there was no interaction between diet treatment and foraging background (Two-Factor ANOVA, $F_{3,3} = 0.0366$, $P = 0.9905$).

3.4 Proportion of successful strikes

Across treatment groups, the proportion of successful strikes ranged from 0 to 1 (mean $\pm$ SEM = 0.78 ± 0.03 ; Fig. 3d). There was no significant effect of diet treatment on the proportion of successful strikes (GLM, $\chi^2_3 = 0.7617092$, $P = 0.8586$). Additionally, there was no influence of foraging background (mean $\pm$ SEM, cryptic background = 0.83 ± 0.04 ; mean $\pm$ SEM, conspicuous background = 0.74 ± 0.06 ; GLM, $\chi^2_1 = 0.8452189$, $P = 0.3579$), and there was no interaction between diet treatment and foraging background (GLM, $\chi^2_3 = 0.5233254$, $P = 0.9137$).

3.5 Proportion of prey items consumed

Across treatment groups, the proportion of prey items consumed ranged from 0 to 0.9 (mean $\pm$ SEM = 0.48 ± 0.03 ; Fig. 3e). There was no significant effect of diet treatment on the proportion of prey items consumed by frogs (GLM, $\chi^2_3 = 0.2875761$, $P = 0.9623$). Additionally, there was no influence of foraging background (mean $\pm$ SEM, cryptic background = 0.52 ± 0.03 ; mean $\pm$ SEM, conspicuous background = 0.44 ± 0.04 ; GLM, $\chi^2_1 = 0.4422832$, $P = 0.5060$), and there was no interaction between diet treatment and foraging background (GLM, $\chi^2_3 = 0.2358666$, $P = 0.9716$).

3.6 Number of pedal luring events

Across treatment groups, the number of pedal luring events ranged from 0 to 26 (mean $\pm$ SEM = 1.88 ± 0.33 ; Fig. 3f). There was no significant effect of diet treatment on the number of pedal luring events displayed by frogs (GLM, $\chi^2_3 = 0.9068761$, $P = 0.8238$). Additionally, there was no interaction between diet treatment and foraging background (GLM, $\chi^2_3 = 0.7587145$, $P = 0.8593$). However, frogs on conspicuous backgrounds did display significantly more pedal luring events compared to those on cryptic backgrounds (mean $\pm$ SEM, cryptic background = 1.09 ± 0.32 ; mean $\pm$ SEM, conspicuous background = 3.84 ± 1.05 , GLM, $\chi^2_1 = 8.4868742$, $P = 0.0036$).

4. Discussion

The aim of this study was to test two competing hypotheses (the Environmental Matching Hypothesis and the Silver Spoon Hypothesis) regarding the influence of carotenoid availability at different life-stages on the foraging performance of *Pseudophryne corroboree*. This was achieved by manipulating dietary carotenoid availability during development and adulthood and quantifying the influence of diet treatment on foraging performance. Our results show no effect of carotenoid supplementation on several measures of foraging performance, including time to first movement towards a prey item, number of stalking events, proportion of time spent actively foraging, proportion of successful strikes, proportion of prey items consumed and the number of pedal luring events. Interestingly, however, there was a significant effect of foraging background on behaviour, whereby frogs on conspicuous backgrounds took longer to first move towards a prey item and also displayed significantly more pedal luring events. This effect indicates that Southern Corroboree frogs might adjust their foraging strategies depending on the degree of prey visibility. Specifically, our results imply that frogs are more likely to adopt a sit and wait foraging strategy incorporating aggressive mimicry (pedal luring) when prey are more visible. This finding is consistent with past studies of foraging behaviour in visual predators which have reported that optical cues and motion perception are critical for eliciting predatory responses (Radcliffe et al., 1986; Hagman and Shine, 2008; McFadden et al., 2010). Furthermore, this finding is consistent with the belief that toe twitching in *P. corroboree* functions as a visual lure (McFadden et al., 2010), as opposed to producing vibrational stimuli that trigger prey movement and improve prey detectability, as thought to be the case in other anurans (Sloggett and Zeilstra, 2008).

The findings of this study do not provide any evidence that dietary carotenoids improve the visual acuity of *P. corroboree*, as has been reported in insect and bird species (Ragusso et al., 2007; Goyret et al., 2009; Toomey and McGraw, 2011). Furthermore, these outcomes were not in the direction predicted by either the Environmental Matching or Silver Spoon Hypotheses (Monaghan, 2008). There are several possible explanations as to why carotenoid supplementation did not have the

predicted beneficial effect on foraging performance. First, the methods we used may not have been appropriate to detect benefits of carotenoid supplementation. All of our trials were conducted in full light, but carotenoids may only improve visual acuity in *P. corroboree* under low light conditions. Indeed, there is some evidence in birds that experimental lighting conditions can influence the effect of carotenoid supplementation on foraging performance (Toomey and McGraw, 2011). It is also important to recognise that our trials were conducted in small arenas, which may have increased the frequency of chance encounters between frogs and prey. If arenas were larger and interactions with prey were less frequent, positive effects of carotenoids on vision and foraging performance may have been apparent. Additionally, it is important to note that our tests did not involve natural prey items or natural substrates. If we used prey that had coevolved with toadlets (e.g. alpine ants), and we presented these on natural substrates (e.g. sphagnum moss), we may have detected a significant effect of dietary carotenoids on foraging performance. In brief, carotenoid-related improvements to vision and foraging performance may only become evident when animals are sufficiently challenged. As such, future investigations aiming to test whether dietary carotenoids improve foraging performance in *P. corroboree* should measure performance under a broader range of contexts.

An alternative reason why carotenoids did not influence foraging performance is that Southern Corroboree frogs lack the capacity to utilise dietary carotenoids, as appears to be the case for some other vertebrate species (Costantini et al., 2007; Huggins et al., 2010). An inability to utilise dietary carotenoids is expected for species that have had limited access to dietary carotenoids and have not evolved the cellular machinery (i.e. lipoproteins in the plasma and intestinal mucosa of the gastrointestinal tract) required to process these compounds (Parker, 1996). However, this does not seem to be the case for *P. corroboree* because the diet of this species is known to consist of algae, detritus and insects (Osborne, 1991), all of which contain high concentrations of carotenoids (Lichtenthaler, 1987; Eeva et al., 2010). Furthermore, recent research in *P. corroboree* has indicated that dietary carotenoids can significantly enhance skin colouration (Byrne and Silla, unpublished data) and escape performance (Silla et al., 2016), indicating that *P. corroboree* is capable of efficiently processing and assimilating dietary carotenoids. To conclusively demonstrate that the experimental animals were indeed assimilating dietary carotenoids it would have been necessary to quantify carotenoid levels in either plasma, retina or other tissues, as achieved by past studies investigating the effects of dietary carotenoids on fitness-determining traits in various vertebrate groups (Piercy et al., 2000; Lin et al., 2010; Toomey and McGraw, 2011; Giraudeau et al., 2013; Wawrzyniak et al., 2013). In the present study, such invasive procedures were not possible for ethical reasons due to the high conservation value of our study species, which is listed as critically endangered at both a national and international level.

A more plausible explanation for our results is that the carotenoid dose administered may have been sub-optimal for this species. To date, the effect of carotenoid dose on foraging performance has

not been systematically determined for any species (Ragusso et al., 2007; Goyret et al., 2009; Li et al., 2009; Toomey and McGraw, 2011). However, there is evidence to suggest that dietary carotenoid dose can influence other fitness-determining traits in vertebrates. For example, in rainbow trout fed various concentrations (0.0, 12.5, 25.0, 50.0, 100.0 and 200.0 mg/kg) of the carotenoids astaxanthin and canthaxanthin over a period of six weeks, the colouration (pigmentation) of fish increased linearly with carotenoid concentration (Choubert and Storebakken, 1989). To conclusively determine the effect of carotenoid dose on behavioural performance in *P. corroboree*, a dose-response experiment will be required. Such research will help to identify whether there is a critical dose at which carotenoids decrease oxidative stress and become effective in aiding vision and foraging performance.

Finally, carotenoids may not have influenced foraging performance in *P. corroboree* if they were preferentially invested in other traits, resulting in a developmental trade-off among traits (Bertrand et al., 2006; Lin et al., 2010; Toomey et al., 2010). The carotenoid trade-off hypothesis predicts that when carotenoids are a limiting resource, individuals must allocate carotenoids to traits that have the most significant impact on fitness (Lin et al., 2010). Several studies in fish and birds have shown that when dietary carotenoids are in short supply, these compounds are preferentially invested into traits that directly influence physiological functioning and survival (Clotfelter et al., 2007; Bertrand et al., 2006; Benito et al., 2011; Moreno-Rueda, 2011). For instance, supplemented fighting fish (*Betta splendens*) have been shown to preferentially invest carotenoids into immune response and health before ornamental colouration (Clotfelter et al., 2007). In *P. corroboree*, it is known that dietary carotenoids are invested in escape performance and skin colouration (Silla et al., 2016; Byrne and Silla, unpublished data), however, the fitness advantage of such investment remains unknown. The fitness benefit of investing carotenoids in skin colour or muscle performance may far outweigh any benefits associated with investing carotenoids in vision. If this is true, carotenoids may only be invested in traits that impact vision when excess carotenoids are freely available. This idea could be tested experimentally by manipulating carotenoid availability and quantifying how carotenoids are partitioned among different traits (i.e. colouration, immune function, growth and development, behavioural performance and visual acuity).

5. Conclusion

In conclusion, the aim of our study was to investigate how carotenoid availability at different life-stages influences the foraging performance of *Pseudophryne corroboree*. Carotenoid supplementation at any life-stage was not found to improve foraging performance, providing no support for either the Environmental Matching or the Silver Spoon Hypotheses. Dietary carotenoids may not have influenced foraging performance in *P. corroboree* because the dose administered was sub-optimal, or because trade-offs among traits may have resulted in carotenoids being preferentially invested in traits other than foraging performance. Consequently, continued investigation will be

required to conclusively determine the role of dietary carotenoids at different life-stages on the fitness of *P. corroboree*. This work is needed as there remains a dearth of information concerning the influence of dietary carotenoids received at different life-stages on behaviour in amphibians and other vertebrate groups. Gaining this information will greatly improve our understanding of the influence of diet and nutrition at different life-stages on individual phenotype and fitness.

Acknowledgements

We are grateful to staff in the Herpetology Department of Melbourne Zoo for providing the Southern Corroboree Frog eggs employed in the present study. We thank Taronga Zoo's Herpetofauna division, in particular Adam Skidmore, Michael McFadden and Dr Peter Harlow, for providing assistance preparing our captive facilities and offering advice on captive husbandry requirements. Joseph Bailey is acknowledged for his contribution toward routine frog husbandry and cricket breeding. Additionally, we acknowledge the support of NSW Office of Environment and Heritage (OEH), Threatened Species Officer Dr David Hunter, who facilitates the integration of *ex situ* and *in situ* conservation efforts for this species. This study was funded by the Australian Research Council (Linkage Grant LP140100808), NSW Environmental Trust (grant no. 2012/ RD/0105) and the School of Biological Sciences, University of Wollongong.

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