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Carotenoid supplementation affects the post-hibernation performance of southern corroboree frogs

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Abstract

© 2020 Copyright 2020 by Koninklijke Brill NV, Leiden, The Netherlands. Many animals hibernate to survive winter conditions, however, arousal from hibernation generates reactive oxygen species (ROS) that can cause oxidative stress. Dietary antioxidants, like carotenoids, may reduce oxidative stress during arousal from hibernation, and assist with post-hibernation recovery and performance. We tested the effect of carotenoid supplementation on exercise performance (escape-response and activity) in southern corroboree frogs (Pseudophryne corroboree) following initial arousal from hibernation (24-48 h post-arousal) and post-recovery (six weeks post-hibernation). Carotenoids did not affect performance following initial arousal. However, carotenoids improved escape-response six weeks post-hibernation, with carotenoid-supplemented frogs hopping faster and further in their first hop than unsupplemented frogs. Carotenoids also affected post-recovery activity, with carotenoid-supplemented frogs being less mobile than unsupplemented frogs. Carotenoids may affect post-hibernation performance by reducing oxidative stress or by increasing diet quality. Our study provides novel evidence for an effect of carotenoids on performance post-hibernation and highlights the importance of nutrition to hibernating organisms.

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Carotenoid supplementation affects the post-hibernation performance of southern corroboree frogs

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Summary

Many animals hibernate to survive winter conditions, however, arousal from hibernation generates reactive oxygen species (ROS) that can cause oxidative stress. Dietary antioxidants, like carotenoids, may reduce oxidative stress during arousal from hibernation, and assist with post-hibernation recovery and performance. We tested the effect of carotenoid supplementation on exercise performance (escape-response and activity) in southern corroboree frogs (*Pseudophryne corroboree*) following initial arousal from hibernation (24-48 h post-arousal) and post-recovery (six weeks post-hibernation). Carotenoids did not affect performance following initial arousal. However, carotenoids improved escape-response six weeks post-recovery, with carotenoid-supplemented frogs hopping faster and further in their first hop than unsupplemented frogs. Carotenoids also affected post-recovery activity, with carotenoid-supplemented frogs being less mobile than unsupplemented frogs. Carotenoids may affect post-hibernation performance by reducing oxidative stress or by increasing diet quality. Our study provides novel evidence for an effect of carotenoids on performance post-hibernation and highlights the importance of nutrition to hibernating organisms.

Keywords: anuran, activity, antioxidants, carotenoids, escape-response, hibernation

1. Introduction

Hibernation is a strategy adopted by many animals to enable them to endure periods of extremely low winter temperatures (St-Pierre & Boutilier, 2001; Bagnyukova et al., 2003). During hibernation, individuals supress their metabolic rate, oxygen consumption, and activity, allowing them to survive extended periods without feeding (St-Pierre & Boutilier, 2001). While hibernation is essential for the survival of many species, arousal from hibernation can cause significant physiological stress (Hermes-Lima et al., 2015). High oxygen intake needed to return to a normal metabolic rate, combined with increased energetic expense required to cope with elevated post-hibernation temperatures, generates a large amount of reactive oxygen species (ROS) that can harm the body (Bagnyukova et al., 2003; Hermes-Lima et al., 2015). ROS are radical molecules produced as a by-product of mitochondrial respiration which can oxidise otherwise stable cellular molecules (Powers et al., 2004). When the rate of ROS production exceeds the rate of ROS detoxification, the result is oxidative stress, which can cause irreversible damage to lipids, proteins, DNA, and organismal performance (Powers et al., 2004). During arousal from hibernation, an individual's antioxidant system (which includes both endogenous and exogenous antioxidants) functions to reduce oxidative damage (Hermes-Lima et al., 2015). While it is widely acknowledged that most hibernating animals have evolved the ability to increase endogenous antioxidant levels during arousal from hibernation (termed 'preparation for oxidative stress') (Hermes-Lima et al., 2015), many still experience higher than normal rates of oxidative stress (Bagnyukova et al., 2003; Niu et al., 2018).

A diet rich in exogenous antioxidants, received prior to hibernation, may improve the antioxidant systems capacity to lessen the effects of ROS-induced damage following hibernation. One group of exogenous antioxidants obtained by animals through their diet are

carotenoids, a group of over 750 hydrocarbon compounds synthesised by photosynthetic plants, bacteria, and fungi (Svensson & Wong, 2011). Dietary carotenoids are consumed by a diversity of animals and may strongly support post-hibernation exercise performance, encompassing traits such as escape-response and general activity, which are known to directly impact survival (Edmunds, 1974). Post-hibernation escape-response behaviour and activity are expected to dictate how likely an individual is to find resources (such as food and refuge) and escape predation following arousal. However, these behaviours can be energetically costly and elevate the production of ROS (Powers et al., 2004; Blount & Matheson, 2006; Rowe et al., 2015). Increased consumption of carotenoids prior to hibernation is expected to reduce the stress experienced by individuals when exhibiting these behaviours post-hibernation, and, in turn, improve performance (Powers et al., 2004; Blount & Matheson, 2006).

Beyond performance benefits received during arousal form hibernation, consumption of carotenoids may also be critically important during recovery from hibernation. Several studies have shown that ROS-induced stress can be experienced for several months following hibernation (Cooper et al., 1992; Feidantsis et al., 2012; Feidantsis et al., 2013; Hoelzl et al., 2016). Furthermore, individuals emerging from hibernation typically do so in spring, a season associated with rapid somatic growth, and increased investment in reproductive traits and processes; both of which are energetically costly activities known to generate high amounts of ROS (Blount, 2004; Metcalfe & Alonso-Alvarez, 2010). Therefore, if damaging effects of ROS are cumulative (as suggested by Bagnyukova et al., 2003; Metcalfe & Alonso-Alvarez, 2010), dietary antioxidants may become increasingly important in the weeks following arousal. While past studies have shown that exogenous antioxidants can improve performance (Aoi et al., 2003; Blount & Matheson, 2006; Larcombe et al., 2008; Silla et al.,

95 2016), no study to date has investigated how dietary antioxidants influence performance
96 following initial arousal from hibernation and following recovery from hibernation.

97 Anuran amphibians (frogs and toads) present excellent opportunities to test the effects
98 of carotenoid supplementation on post-hibernation performance. Globally, a diversity of
99 anuran species utilise hibernation as an overwintering survival strategy, and the effects of
100 hibernation on antioxidant defences and oxidative stress in anurans has been well
101 documented (Boutilier, 2001; St-Pierre & Boutilier, 2001; Bagnyukova et al., 2003;
102 Feidantsis et al., 2012; Prokic et al., 2017; Niu et al., 2018). Specifically, it has been shown
103 that hibernating anurans can experience higher antioxidant activity than normal both during,
104 and post-hibernation (Bagnyukova et al., 2003), but also that hibernation can increase levels
105 of oxidative stress in heart and muscle tissue (Niu et al., 2018), and reduce muscle
106 performance (Hudson and Franklin, 2002). At present, there is a lack of information
107 regarding the long-term effects of hibernation on anuran performance, however two studies in
108 water frogs (*Pelophylax ridinbundus*) have shown that hibernation can increase the long-term
109 occurrence of heat shock proteins and oxidative stress, which is likely to have direct effects
110 on performance (Feidantsis et al., 2012; Feidantsis et al., 2013).

111 The present study aimed to investigate the effect of dietary carotenoids on the post-
112 hibernation escape-response performance and general activity of southern corroboree frogs
113 (*Pseudophryne corroboree*). *P. corroboree* enter hibernation during the onset of austral
114 winter and evade freezing by burrowing into underground refuges (at the base of tussock
115 grasses, under logs, and in sphagnum bogs) (Osborne, 1991; McFadden, 2019, personal
116 comms). Past studies with *P. corroboree* have shown that dietary carotenoids can improve
117 escape-response performance (Silla et al., 2016; McInerney et al., 2017), and modify
118 exploratory behaviour (Kelleher, et al., 2019). In the present study, we tested the effect of
119 carotenoid supplementation on the post-hibernation performance of adult *P. corroboree*

following initial arousal from hibernation (24-48 hrs post-arousal) and following recovery from hibernation (six weeks post-arousal) to explore whether exogenous antioxidants play a role in post-hibernation performance. We predicted that dietary carotenoids would assist with both initial arousal and recovery from hibernation, such that carotenoid-supplemented individuals would perform significantly better than unsupplemented individuals at both time periods. Further to this, we predicted that the performance of carotenoid supplemented, but not unsupplemented, frogs would improve between initial arousal and recovery from hibernation, reflecting the capacity for carotenoids to aid in post-hibernation recovery over time.

2. Materials and Methods

2.1 Study species

Pseudophryne corroboree is a small (25-30 mm, snout-vent length) anuran (family: Myobatrachidae) endemic to the Snowy Mountain region of New South Wales (Osborne, 1991). In this region, adult frogs enter a state of hibernation during the winter months (Osborne, 1991). *P. corroboree* is characterised by bright yellow and black markings (Osborne, 1991). As with other *Pseudophryne* species, their predominant mode of locomotion is a slow to rapid crawl, although individuals hop short distances when threatened (Colefax, 1956; Silla et al., 2016). *P. corroboree* feed on algae during the larval life-stage (Osborne, 1991), and invertebrates during the adult life-stage (Osborne, 1991), both of which contain carotenoids (Lichtenthaler, 1987).

2.2 Experimental design

To test the effects of carotenoid supplementation on the post-hibernation escape-response performance and general activity of *P. corroboree*, frogs were assigned to one of two dietary treatments from hatching (n = 24 per treatment). These were; (1) an unsupplemented diet containing no added carotenoids, and (2) a carotenoid-supplemented diet, which contained added carotenoids from a broad-spectrum carotenoid mix (and potentially other plant-derived nutrients; Superpig; Rapashy®, CA, USA). To quantify the effect of carotenoid supplementation on escape-response and general activity following hibernation, behavioural assays were conducted over two hibernation cycles that took place annually. Escape-response assays were conducted between November 19, 2015 and January 1, 2016, and activity assays were conducted in the following annual hibernation cycle between November 24, 2016 and January 6, 2017. Details of these behavioural assays can be found in sections 2.5 and 2.6.

2.3 Husbandry and nutrition prior to hibernation

Fertilised *P. corroboree* eggs (n = 48) used in this study were obtained from a captive colony held at Melbourne Zoo, Australia. Eggs were transported to the University of Wollongong on July 19, 2013 and stimulated to hatch via flooding with reverse-osmosis (R.O.) water. During both larval (tadpole) and adult (post-metamorphic) life-stages, animals were housed individually according to husbandry methods stated elsewhere (McInerney et al., 2016; Silla et al., 2016). At the commencement of the experimental period, tadpoles were randomly assigned to either an unsupplemented dietary treatment or a carotenoid-supplemented dietary treatment (N = 24 frogs per treatment). The unsupplemented tadpole dietary treatment consisted of 2 g of ground fish flakes (75:25 mixture of Sera Flora/ Sera Sans) suspended in

20 ml of R.O. water, drawn into syringes, frozen and then thawed prior to use. For the first two months of the experimental period tadpoles received two droplets (range = 0.015 g – 0.018 g dry mass) of homogenised food suspension three times a week. Food supply was then increased to four droplets (range = 0.030 g – 0.036 g dry mass) until metamorphosis (forelimb emergence: Gosner stage 42; Gosner, 1960). The unsupplemented tadpole dietary treatment contained low levels of total carotenoids (0.015 mg g^{-1}) (see Silla et al., 2016). During the tadpole life-stage, the carotenoid-supplemented dietary treatment consisted of the unsupplemented dietary treatment described above, supplemented with 0.040 g of carotenoid mixture containing 20 different carotenoid pigments (Superpig; Rapashy®, CA, USA) (Silla et al., 2016).

During metamorphosis (Gosner stage 42 to 46), individuals were not provided with food as nutritional needs are met by re-absorption of the tail. Upon completion of metamorphosis, frogs received live crickets twice weekly so that food was available *ad libitum*. Frogs in the unsupplemented adult dietary treatment were provided with crickets that had been fed 48 h earlier with granny-smith apple (poor in carotenoids). The unsupplemented adult diet contained very small amounts of total carotenoids (0.005 mg g^{-1}). Frogs in the carotenoid-supplemented dietary treatment were provided with crickets that had been fed 48 h earlier with carrot (rich in carotenoids) and dusted with 1 g of carotenoid mixture (Superpig; Rapashy®, CA, USA). In total, the carotenoid-supplemented adult dietary treatment contained 1.152 mg g^{-1} of carotenoids (see Silla et al., 2016). Once a week, crickets in both dietary treatments were dusted with 0.200 g of calcium powder (Repti-Cal, Aristopet, Australia) prior to feeding to prevent nutrient deficiencies. Crickets ranged in age from 2 – 10 days old for both dietary treatments. Frogs received experimental diets for approximately two and a half years prior to escape-response assays being conducted, and three and a half years prior to activity trials being conducted (see details below).

2.4 Hibernation conditions

To simulate the onset of winter and induce frogs to enter a state of hibernation, the temperature in the experimental room was dropped progressively from 20˚C to 10˚C (Figure 1). In preparation for hibernation, once the temperature was reduced to 13˚C, feeding ceased and frogs were not fed until arousal from hibernation (a total of six weeks; Figure 1). Following the first week of fasting, the temperature was reduced to 10˚C. At this temperature, frogs entered a state of hibernation and displayed very little activity. During this time, the lighting within the experimental room was placed on an 11: 13 h light: dark cycle with a 15 min dusk period to simulate conditions experienced in Kosciusko National Park (in the Snowy Mountains) during winter (June - August) (Bureau of Meteorology, 2015). Following a four-week period at 10℃, temperature was incrementally increased over a two-week period to reach 15˚C, which caused frogs to arouse from hibernation. Initial arousal behavioural assays took place 24- 48 h after temperatures were increased to 15 ˚C (hereafter referred to as trial week 0) (Figure 1). The following day, feeding resumed and frogs were fed their adult dietary treatments for a six-week recovery period before being exposed to a second round of behavioural assays (hereafter referred to as trial week 6) (Figure 1).

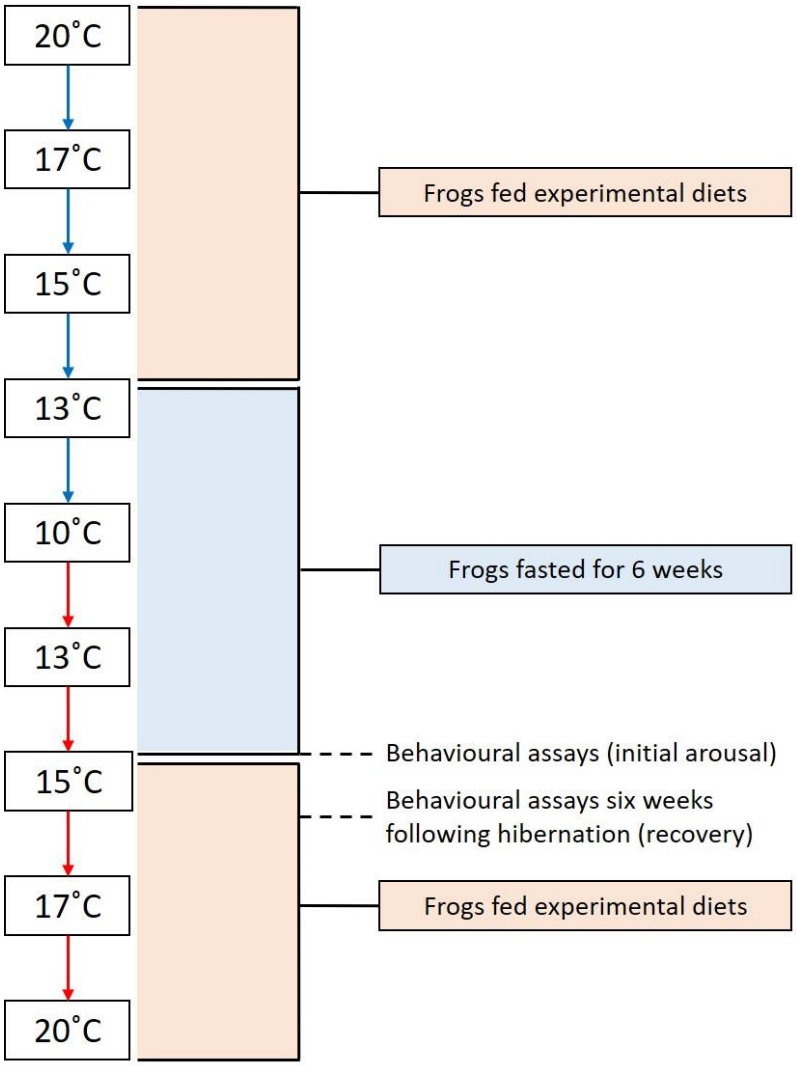


Figure 1. Yearly hibernation cycle for *P. corroboree*. Frogs were unfed at 13℃ and entered a state of hibernation at 10℃. Frogs were aroused from hibernation as temperatures returned to 15℃. All behavioural assays were conducted at 15℃ following hibernation. Initial arousal behavioural assays were conducted 24-48 h following arousal from hibernation when frogs were still fasted. Recovery behavioural assays were conducted six weeks following hibernation when frogs had resumed their experimental diets.

2.5 Escape-response assays

Escape performance trials were conducted on November 19 and 20, 2015 (trial week 0) and on December 31, 2015 and January 1, 2016 (trial week 6). In order to quantify escape-response, frogs were subjected to hopping performance trials where individuals were pursued to the end of a narrow hopping track (30 cm x 4 cm x 4 cm) by a model Alpine Copperhead snake (*Austrelaps ramsayi*), found naturally in the same range as *P. corroboree*. Before trials commenced, frogs were placed in front of the model snake, separated by an opaque divider where they were held by the researcher until they remained motionless. Frogs were approximately 5 cm from the model snake before trials began. The opaque divider was then removed and the model snake moved towards the frog at a constant speed of 0.750 cm s^{-1} via an automated motorised pulley system (Tamiya Corp., Aliso Viejo, CA, USA) controlled by a 12 V 36 RPM motor (Soanar, Sydney, NSW, Australia) attached to a 12 V DC speed controller and Plus Switch mode regulated plug pack (Powertech, Taiwan, China) (Supplementary movie S1). Between trials, the hopping track was wiped clean with ethanol and then R.O. water to remove any chemosignals that may have influenced individuals' performance. An equal number of representatives from each dietary treatment were tested on each trial day, and trial order was randomised to avoid temporal effects. All trials were video recorded using a high definition Sony Exmor R Handycam mounted on a tripod approximately 50 cm above the hopping track. Videos were later viewed using Windows Movie Maker software and the following behavioural response variables were quantified; (1) hopping speed (cm s^{-1}) (of the first hop), (2) length of the first hop (cm), and (3) total hopping distance (cm) in response to the first tap from the snake.

2.6 Activity assays

Activity assays were conducted on November 24 and 25, 2016 (trial week 0) and on January 5 and 6, 2017 (trial week 6). In order to quantify general activity, frogs were moved from their home containers into experimental containers (31.9 cm x 17.8 cm x 20 cm). Each experimental container was lined on the bottom with white corflute, and the walls of the experimental container were lined with black opaque plastic to remove visual contact with neighbouring frogs. Frogs were acclimated to experimental containers under a black opaque plastic cup for five minutes. Following this acclimation period, the cup was removed using a pulley system and the activity behaviour of each individual was video recorded for 60 minutes (Supplementary movie S2). Trials were simultaneously completed in six blocks of eight individuals. Within each block, there were equal representatives from each dietary treatment and trial order was randomised to avoid temporal effects. During trials, the researcher was not present in the room in order to remove any observer effects on the frogs' activity. Between trials, the experimental containers were wiped clean with ethanol and then R.O. water to remove any chemosignals that may have influenced individuals' performance. All trials were video recorded using high definition Panasonic HC-W580M cameras suspended approximately 60 cm above the experimental containers. Videos were later re-watched in Anymaze software and the following response variables were quantified; (1) distance travelled (cm), (2) the number of mobile episodes, defined as the number of times frogs became active during the activity assay, and (3) time mobile (s). Frogs were classified as being immobile after five seconds of inactivity.

2.7 Statistical analysis

For each behavioural assay, neither body size (snout-vent length or mass) nor sex had a significant influence on any of the response variables measured, and body size did not change significantly during behavioural assay periods. Therefore, body size and sex were not included as co-variates in any subsequent statistical analyses. For each behavioural assay, four individuals were excluded from analyses due to deaths within the trial period or unusable behavioural trials (therefore Unsupplemented dietary treatment; n = 21, Carotenoid-supplemented dietary treatment; n = 23). Because data were not normally distributed non-parametric tests were used. To test the effect of dietary treatment on hopping speed, length of the first hop, hopping distance, distance travelled, number of mobile episodes, and time mobile for each trial week (trial week 0 and trial week 6), we ran Wilcoxon rank sum tests. In each model, dietary treatment was the explanatory variable and either hopping speed, length of the first hop, hopping distance, distance travelled, number of mobile episodes, or time mobile was the response variable. To test the effect of trial week (time) on hopping speed, length of the first hop, hopping distance, distance travelled, number of mobile episodes, and time mobile for each dietary treatment, we ran Friedman's tests. In each model, trial week was the explanatory variable, individual ID was the block, and hopping speed, length of the first hop, hopping distance, distance travelled, number of mobile episodes, or time mobile was the response variable. All statistical analyses were performed in R studio 1.1.447.

2.8 Ethics note

All procedures in this experiment were carried out following evaluation and approval from the University of Wollongong's Animal Ethics Committee (approved number AE13/13) following ASAB/ABS ARRIVE guidelines.

3. Results

3.1 Escape-response assays

3.1.1 Hopping speed

Dietary treatment had no significant effect on hopping speed in trial week 0 (mean ± SEM; Unsupplemented dietary treatment = 1.929 ± 0.555 cm s^{-1}, Carotenoid-supplemented dietary treatment = 1.956 ± 0.444 cm s^{-1}) (Wilcoxon: $W = 257.500$, $P = 0.709$) (Figure 2(A)). However, carotenoid-supplemented frogs hopped significantly faster than unsupplemented frogs in trial week 6 (mean ± SEM; Unsupplemented dietary treatment = 2.685 ± 0.623 cm s^{-1}, Carotenoid-supplemented dietary treatment = 5.450 ± 0.861 cm s^{-1}) (Wilcoxon: $W = 338$, $P = 0.023$) (Figure 2(B)). Furthermore, individuals from the carotenoid-supplemented dietary treatment hopped significantly faster in trial week 6 than in trial week 0 (Friedman's test; $x^2_1 = 10.714$, $P = 0.001$), whereas individuals in the unsupplemented dietary treatment did not change in their hopping speed between trial weeks (Friedman's test; $x^2_1 = 0.053$, $P = 0.819$). These results indicate that individuals from the carotenoid-supplemented dietary treatment improved in their hopping performance over the six week post-hibernation recovery period.

3.1.2 Length of the first hop

Dietary treatment had no significant effect on length of the first hop in trial week 0 (mean ± SEM; Unsupplemented dietary treatment = 0.614 ± 0.154 cm, Carotenoid-supplemented dietary treatment = 0.913 ± 0.162 cm) (Wilcoxon: $W = 288.500$, $P = 0.259$) (Figure 2©). However, carotenoid-supplemented frogs hopped significantly further in their first hop than unsupplemented frogs in trial week 6 (mean ± SEM; Unsupplemented dietary treatment = 0.929 ± 0.180 cm, Carotenoid-supplemented dietary treatment = 1.652 ± 0.249 cm) (Wilcoxon: $W = 333$, $P = 0.029$) (Figure 2(D)). Furthermore, individuals from the carotenoid-supplemented dietary treatment hopped significantly further in their first hop in trial week 6 than in trial week 0 (Friedman's test; $x^2_1 = 8.895$, $P = 0.003$), whereas individuals in the unsupplemented dietary treatment did not change in their hopping speed between trial weeks (Friedman's test; $x^2_1 = 0.529$, $P = 0.467$).

3.1.3 Hopping distance

Dietary treatment had no significant effect on hopping distance in trial week 0 (mean ± SEM; Unsupplemented dietary treatment = 1.724 ± 0.453 cm, Carotenoid-supplemented dietary treatment = 1.022 ± 0.19 cm) (Wilcoxon: $W = 219.500$, $P = 0.603$) (Figure 2(E)). While carotenoid-supplemented individuals hopped further than unsupplemented individuals in trial week 6 (mean ± SEM; Unsupplemented dietary treatment = 2.881 ± 0.100 cm, Carotenoid-supplemented dietary treatment = 4.696 ± 1.100 cm) (Figure 2(F)), this was marginally un-significant (Wilcoxon: $W = 322$, $P = 0.058$). Individuals from the carotenoid-supplemented dietary treatment hopped significantly further in trial week 6 than in trial week 0 (Friedman's test; $x^2_1 = 13.762$, $P < 0.001$), whereas individuals in the unsupplemented dietary treatment

did not change in their hopping speed between trial weeks (Friedman's test; $x^2_1 = 1.471$, $P = 0.225$).

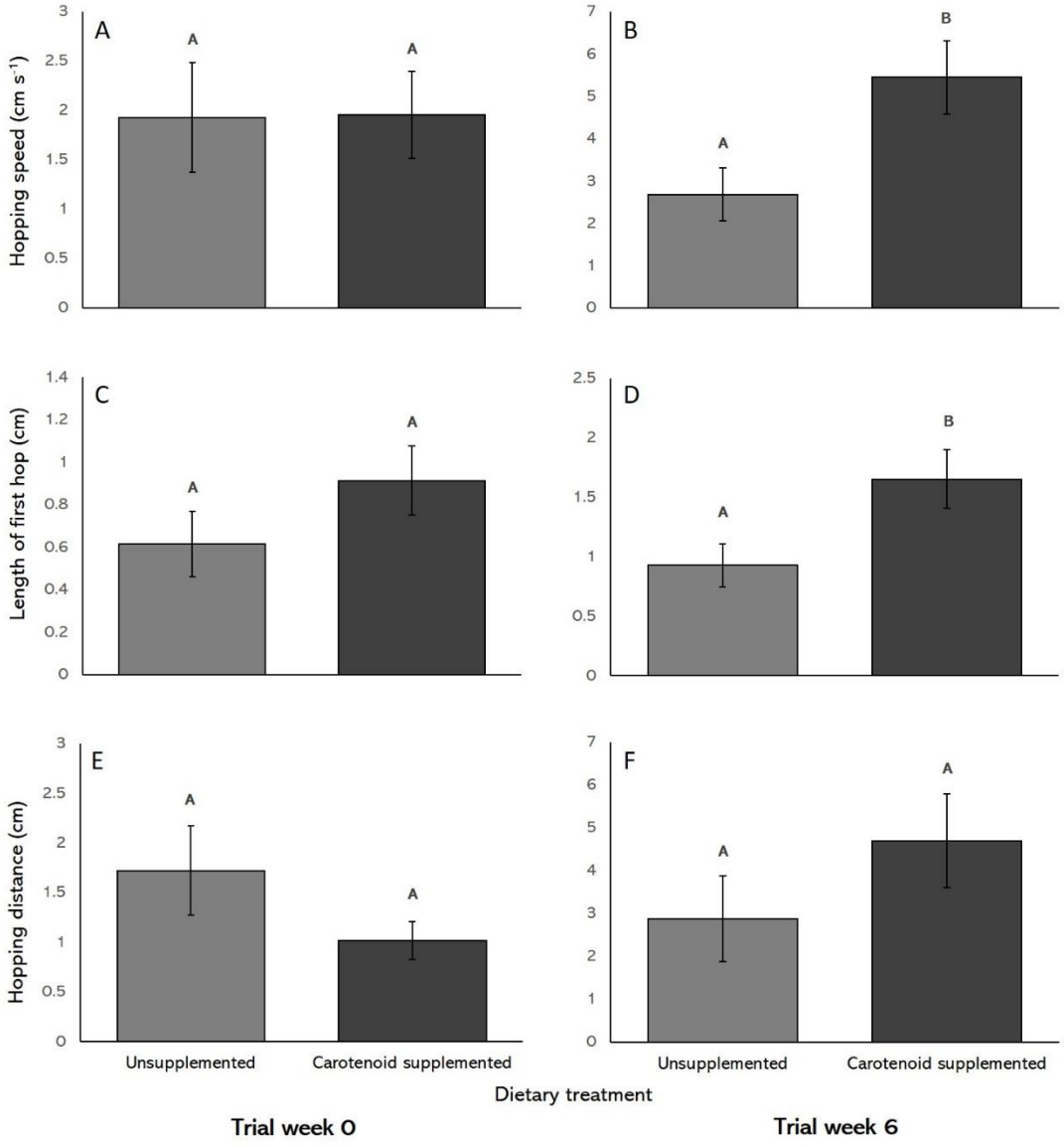


Figure 2. Effect of carotenoid supplementation on; hopping speed (cm s^{-1}) in (A) trial week 0 and (B) trial week 6, length of the first hop (cm) in (C) trial week 0 and (D) trial week 6, and hopping distance (cm) in (E) trial week 0 and (F) trial week 6 for *P. corroboree*. Dietary treatments were; Unsupplemented (n = 21) or Carotenoid-supplemented (n = 23). Data shown are untransformed means ± SEM. Columns not connected by a letter are significantly different.

3.2 Activity assays

3.2.1 Distance travelled

Dietary treatment had no significant effect on the distance travelled in trial week 0 (mean ± SEM; Unsupplemented dietary treatment = 612.891 ± 83.479 cm, Carotenoid-supplemented dietary treatment = 655.739 ± 106.375 cm) (Wilcoxon: $W = 236$, $P = 0.907$) (Figure 3(A)), or trial week 6 (mean ± SEM; Unsupplemented dietary treatment = 7.322 ± 136.651 cm, Carotenoid-supplemented dietary treatment = 521.413 ± 65.917 cm) (Wilcoxon: $W = 192$, $P = 0.252$) (Figure 3(B)). Furthermore, there was no effect of trial week on the distance travelled by individuals in the carotenoid-supplemented dietary treatment (Friedman's test; $x^2_1 = 0.043$, $P = 0.835$), or the unsupplemented dietary treatment (Friedman's test; $x^2_1 = 0.048$, $P = 0.827$).

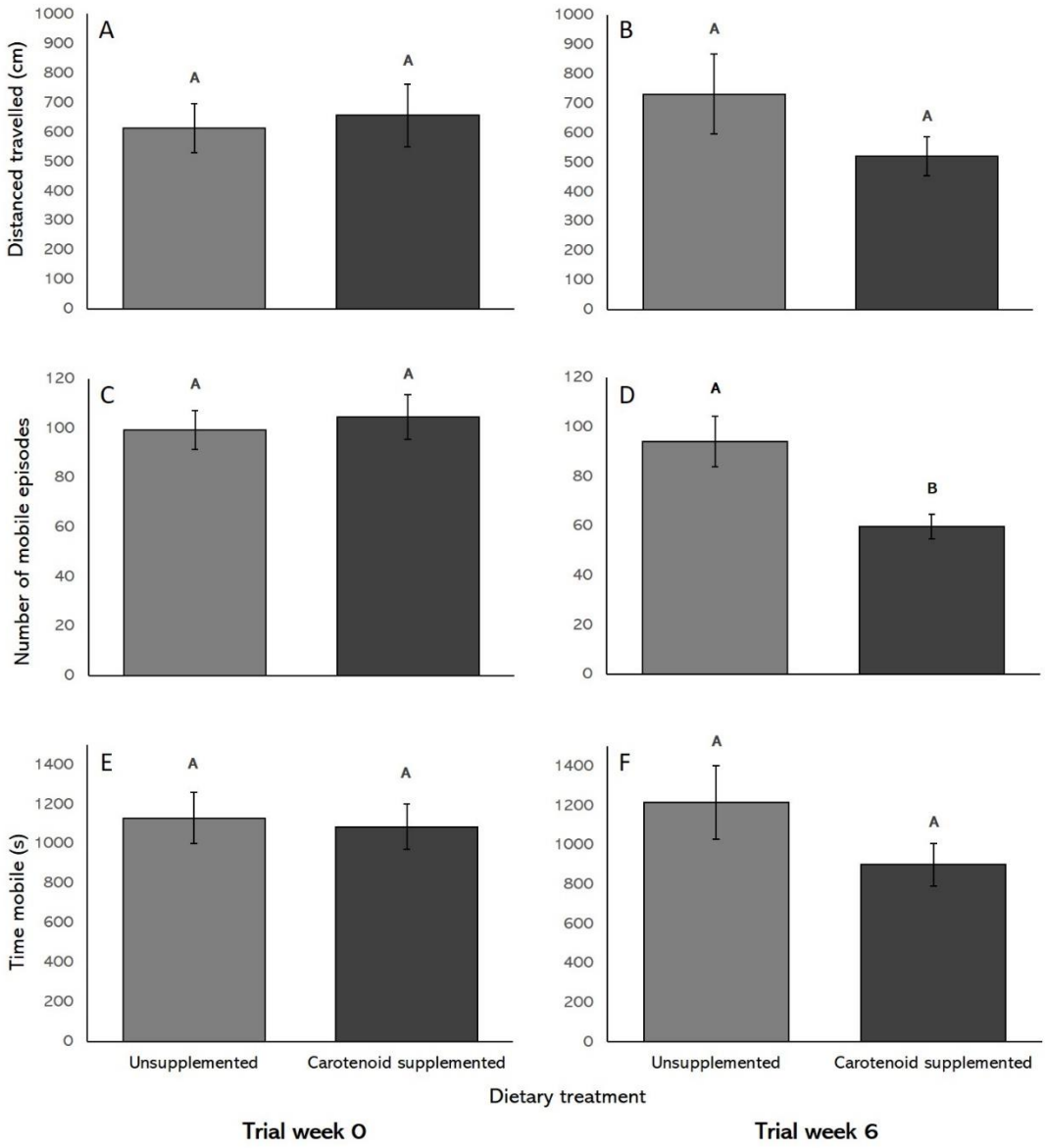
3.2.2 Number of mobile episodes

Dietary treatment had no significant effect on the number of mobile episodes in trial week 0 (mean ± SEM; Unsupplemented dietary treatment = 99.238 ± 7.979, Carotenoid-supplemented dietary treatment = 104.478 ± 9.142) (Wilcoxon: $W = 263.500$, $P = 0.613$) (Figure 3©). However, unsupplemented frogs had significantly more mobile episodes than carotenoid-supplemented frogs in trial week 6 (mean ± SEM; Unsupplemented dietary treatment = 94.143 ± 10.171, Carotenoid-supplemented dietary treatment = 59.826 ± 5.005) (Wilcoxon: $W = 98$, $P < 0.001$) (Figure 3(D)). Furthermore, individuals from the carotenoid-supplemented dietary treatment had significantly fewer mobile episodes in trial week 6 than in trial week 0 (Friedman's test; $x^2_1 = 15.696$, $P < 0.001$), whereas individuals in the

unsupplemented dietary treatment did not change in the number of mobile episodes they exhibited between trial weeks (Friedman's test; $x^2_1 = 0.048$, $P = 0.827$).

3.2.3 Time mobile

Dietary treatment had no significant effect on the time mobile in trial week 0 (mean ± SEM; Unsupplemented dietary treatment = 1128.719 ± 129.525 s, Carotenoid-supplemented dietary treatment = 1086.191 ± 115.390 s) (Wilcoxon: $W = 237$, $P = 0.926$) (Figure 3(E)), or trial week 6 (mean ± SEM; Unsupplemented dietary treatment = 1215.743 ± 187.797 s, Carotenoid-supplemented dietary treatment = 899.809 ± 107.607 s) (Wilcoxon: $W = 187$, $P = 0.207$) (Figure 3(F)). Furthermore, there was no effect of trial week on the time mobile for individuals in the carotenoid-supplemented dietary treatment (Friedman's test; $x^2_1 = 0.043$, $P = 0.835$), or the unsupplemented dietary treatment (Friedman's test; $x^2_1 = 0.048$, $P = 0.827$).



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399 **Figure 3.** Effect of carotenoid supplementation on the; distance travelled in (A) trial week 0
400 and (B) trial week 6, number of mobile episodes in (C) trial week 0 and (D) trial week 6, and
401 time mobile (s) in (E) trial week 0 and (F) trial week 6 for *P. corroboree*. Dietary treatments
402 were; Unsupplemented (n = 21) or Carotenoid-supplemented (n = 23). Data shown are
403 untransformed means ± SEM. Columns not connected by a letter are significantly different.

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4. Discussion

While hibernation is essential for the survival of many organisms, high levels of reactive oxygen species (ROS), generated during arousal from hibernation, are expected to negatively impact post-hibernation performance. Dietary supplementation with exogenous antioxidants, such as carotenoids, is expected to limit the degree of oxidative stress experienced during arousal from hibernation, and aid in post-hibernation recovery and performance. Both escape-response and general activity are critical for the survival of individuals following hibernation but can cause significant oxidative stress. In this study, we tested the effect of dietary carotenoids on the post-hibernation escape-response performance and general activity of the southern corroboree frog, *Pseudophryne corroboree*. Individuals were fed either a carotenoid-supplemented or unsupplemented dietary treatment and their escape-response and general activity were assayed following initial arousal from hibernation (24-48 hrs post-arousal; trial week 0) and following recovery from hibernation (six weeks post-arousal; trial week 6). In regard to escape-response, carotenoid supplementation had no effect on any measure of performance (hopping speed, length of the first hop, or hopping distance) in trial week 0. However, the escape-response performance of carotenoid-supplemented individuals significantly improved over time, while the performance of unsupplemented individuals remained unchanged. Overall, by trial week 6, carotenoid-supplemented individuals outperformed unsupplemented individuals with regard to their hopping speed, and length of the first hop, but not hopping distance. In regard to activity, there was no significant difference in performance between dietary treatments in trial week 0 (distance travelled, number of mobile episodes, or time mobile). However, by trial week 6, carotenoid-supplemented individuals had fewer mobile episodes than unsupplemented individuals during trials and had significantly fewer mobile episodes over time. These results indicate that

carotenoid supplementation did not improve performance following initial arousal from hibernation, but that dietary carotenoids enhanced escape-response performance and significantly influenced activity following recovery from hibernation.

Unexpectedly, our results showed that there was no effect of carotenoid supplementation on either the escape-response performance or general activity of frogs following arousal from hibernation (trial week 0). One reason for this might be that the carotenoid stores of frogs were depleted during hibernation. While hibernation involves severe metabolic depression to reduce the likelihood of starvation, nutrient stores within the body can still be metabolised (Boutilier, 2001). For instance, European common frogs, *Rana temporaria*, only supress their metabolic rate by 50% during hibernation, and when exposed to more than three weeks of hibernation, rapidly consume their glycogen and carbohydrate stores (Boutilier, 2001). It is not known whether frogs deplete carotenoid stores during hibernation, but this outcome has been reported in other ectotherms that undergo estivation. For example, *Helisoma trivolvis* snails have been found to have a significant reduction in their lutein store during periods of estivation (metabolic depression during extended dry periods) (Arthur et al., 2006). An alternative explanation for our results is that the generation of ROS during arousal from hibernation may have been too great for the carotenoid dose administered to have a beneficial effect. Arousal from hibernation is a physiologically challenging event that generates large amounts of ROS (Bagnyukova et al., 2003; Niu et al., 2018). When ROS production exceeds the capacity of the antioxidants system to quench and stabilise it, oxidative stress results (Powers et al., 2004). Previously, arousal from hibernation has been shown to cause oxidative damage to lipids, heart tissue, and gastrocnemius muscle tissue in anuran amphibians (Bagnyukova et al., 2003; Niu et al., 2018). Therefore, it is possible that frogs in our study experienced similar damaging affects following arousal from hibernation.

Another explanation for why we did not detect an effect of carotenoids on either escape-response performance or general activity in trial week 0 may be that carotenoids were not taken up or assimilated prior to hibernation. However, this seems highly unlikely because frogs in the present study received carotenoids for several years prior to testing. Moreover, a previous study with these animals demonstrated that carotenoid supplementation improves escape-response performance prior to hibernation (Silla et al., 2016). Therefore, an alternative, and more plausible, explanation may be that supplementation with carotenoids limited the upregulation of endogenous antioxidants. During times of stress, ROS generation can trigger the upregulation of the endogenous antioxidants system, which helps to reduce oxidative damage (Oztasan et al., 2004; Peternelj & Coombes, 2011). In preparation for the overproduction of ROS associated with arousal from hibernation, many species have evolved the ability to produce high levels of endogenous antioxidants (deemed 'preparation for stress') (Hermes-Lima et al., 1998; Hermes-Lima et al., 2015). However, it has been suggested that supplementation with exogenous antioxidants can limit the performance of the endogenous antioxidant system and, in some cases, even result in a greater incidence of oxidative damage (Peternelj & Coombes, 2011). While this theory remains to be tested during arousal from hibernation, it would explain why the escape-response performance of carotenoid-supplemented individuals did not differ from unsupplemented individuals immediately after arousal. To conclusively determine the effect of ROS, endogenous antioxidants, and dietary carotenoids on post-hibernation performance, circulating antioxidants and markers of oxidative stress in target tissue and blood would need to be quantified. This was not possible in the present study due to the critically endangered status of *P. corroboree*. However, conducting this work in other less vulnerable overwintering anurans would provide important insights into the relative roles of the endogenous and exogenous antioxidants in mitigating oxidative stress prior to, and following, hibernation.

Results from our study showed no effect of carotenoid supplementation on the performance of individuals immediately following arousal from hibernation, but carotenoid supplementation did assist in the post-hibernation recovery of individuals. Our results showed that carotenoid-supplemented individuals improved in their escape-response performance over the recovery period and outperformed unsupplemented individuals in trial week 6 (when considering hopping speed, and length of the first hop). This finding supports our hypothesis that carotenoids may act as antioxidants during recovery from hibernation, and during escape-response episodes following recovery. Arousal from hibernation can generate significant ROS and result in oxidative stress that can have lasting negative effects (Feidantsis et al., 2012; Feidantsis et al., 2013). As antioxidants, carotenoids might help to reduce ROS-induced stress experienced during recovery from hibernation, resulting in better performance. To date, only two studies have directly measured long-term cell and DNA damage caused by hibernation, but both have found that negative effects of hibernation can last for several months (Feidantsis et al., 2013; Hoelzl et al., 2016). To conclusively determine whether carotenoids act as antioxidants to reduce cell damage during recovery from hibernation, future studies would benefit from measuring ROS generation, oxidative stress, and circulating carotenoid levels.

Until such work is conducted, it is possible that there might be alternative explanations for the carotenoid-mediated benefit to performance we have reported. One possibility is that carotenoids help to improve the general nutritional quality of the diet (Ogilvy & Preziosi, 2012), which may directly affect the recovery of individuals. Specifically, carotenoid supplementation may allow more resources, such as lipids and proteins, to be allocated to bodily functions that improve the condition of individuals following hibernation- a time typically associated with rapid somatic growth and preparation for reproduction. Another possibility is that the positive effect of supplementation was linked to the presence of other

beneficial compounds in the supplement, as this was a plant extract mix containing several unknown compounds. To eliminate any potential for effects causes by unknown performance enhancers, we recommend that future studies supplement diets with pure carotenoids.

Results from the activity behavioural assay showed that the activity of carotenoid-supplemented individuals decreased over the post-hibernation recovery period (when considering number of mobile episodes). By contrast, unsupplemented individuals did not differ in their activity between trial week 0 and trial week 6. As a result, the number of mobile episodes was significantly lower in carotenoid-supplemented individuals compared to unsupplemented individuals in trial week 6. This result was unexpected, because, due to their antioxidant properties, carotenoids were predicted to increase the post-hibernation activity levels of frogs. Unsupplemented individuals may have exhibited more mobile episodes than carotenoid-supplemented individuals following post-hibernation recovery because they needed to increase their foraging intensity to satiate their elevated metabolic requirements (Weimerskirch et al., 2003). It is believed that most animals will maximise foraging efficiency to invest in costly traits (e.g. somatic growth, development, ornamentation), while also conserving energy (Weimerskirch et al., 2003). However, when an individual's nutritional state does not meet the requirements for maintaining costly traits, they may need to increase their foraging activity (Weimerskirch et al., 2003). In the present study, individuals fed carotenoid-supplemented diets may have had fewer mobile episodes in trial week 6 because they had access to a higher quality diet which met their metabolic demands during recovery. By comparison, unsupplemented individuals may have been more active as they needed to spend more time foraging to meet their metabolic demands. To determine whether carotenoids improve foraging efficiency, future studies should quantify how carotenoid supplementation influences foraging dynamics. It would be particularly

532 informative to investigate how different doses of carotenoids influence metabolic demands
533 and how any associated physiological changes influence foraging efficiency.

534 More broadly, the findings of our study advance our understanding of the role of
535 exogenous antioxidants in organismal functioning following hibernation. Past studies
536 concerning the importance of antioxidants following hibernation have centred on
537 understanding the role of endogenous antioxidants on post-hibernation stress. These studies
538 have found that individuals can increase their endogenous antioxidant capacity to prepare for
539 the stress of arousal from hibernation (see Hermes-Lima et al., 2015), but they may still
540 experience some oxidative damage during arousal and recovery from hibernation
541 (Bagnyukova et al., 2003; Feidantsis et al., 2013; Hoelzl et al., 2016; Niu et al., 2018). Our
542 study is the first to demonstrate a positive influence of exogenous antioxidants on the
543 performance of individuals following hibernation, drawing attention to the possibility that
544 dietary antioxidants are more important to species that undergo hibernation than currently
545 realised. Studies in various hibernating species are now needed to ascertain the general
546 benefit of exogenous antioxidants to post-hibernation performance. Knowledge in this area
547 will deepen our understanding of the links between nutrition and performance.

548 In conclusion, the aim of this study was to investigate the effect of carotenoid
549 supplementation on the post-hibernation performance of the southern corroboree frog, *P.*
550 *corroboree*. We found no effect of carotenoid supplementation on the performance of frogs
551 immediately following arousal from hibernation, however, carotenoid supplementation
552 improved escape-response performance and significantly influenced activity six weeks post-
553 recovery. Investigating the extent to which other species rely on dietary antioxidants to
554 maintain post-hibernation performance will advance our understanding of the nutritional
555 needs of hibernating organisms.
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Supplementary material

Supplementary material is available at *Behaviour* online.

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