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# The effect of dietary antioxidants and exercise training on the escape performance of Southern Corroboree frogs

Emma McInerney

*University of Wollongong*, [epm509@uowmail.edu.au](mailto:epm509@uowmail.edu.au)

Phillip G. Byrne

*University of Wollongong*, [pbyrne@uow.edu.au](mailto:pbyrne@uow.edu.au)

Aimee J. Silla

*University of Wollongong*, [asilla@uow.edu.au](mailto:asilla@uow.edu.au)

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# The effect of dietary antioxidants and exercise training on the escape performance of Southern Corroboree frogs

## **Abstract**

Escape-response behaviour is essential to ensure an individual's survival during a predator attack, however, these behaviours are energetically costly and may cause oxidative stress. Oxidative stress can be reduced by supplementing an individual's diet with exogenous antioxidants or through regular moderate exercise training, which stimulates the upregulation of the endogenous antioxidant system. Two studies have tested the simultaneous effects of dietary antioxidant supplementation and exercise training on animal escape-response behaviour. The present study investigated the effects of dietary carotenoids and exercise training on the escape-response behaviour of Southern Corroboree frogs. Frogs were fed either a carotenoid-supplemented or unsupplemented diet and were exposed to repeated escape-response trials (training) for five consecutive weeks. Carotenoid-supplemented individuals outperformed unsupplemented individuals in initial hopping speed, length of the first hop and hopping distance, however, the performance of frogs in each treatment group became statistically similar after training. Within treatment groups, exercise training significantly improved the hopping speed of unsupplemented frogs, with speeds almost doubling between training weeks one and five. By contrast, exercise training did not significantly improve the hopping speed of carotenoid-supplemented frogs. Our results provide some of the first evidence that exercise training improves escape performance, and that dietary antioxidants may inhibit training-induced benefits.

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The effect of dietary antioxidants and exercise training on the escape performance of  
Southern Corroboree frogs

Emma P. McInerney<sup>1\*</sup>, Phillip G. Byrne<sup>1</sup>, Aimee J. Silla<sup>1</sup>

\*Corresponding author: [epm509@uowmail.edu.au](mailto:epm509@uowmail.edu.au)

Ph: +61 2 4298 1932

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

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Escape-response behaviour is essential to ensure an individual's survival during a predator attack, however these behaviours are energetically costly and place the body under oxidative stress. Oxidative stress can be reduced by supplementing individuals with dietary antioxidants (exogenous antioxidants) or through regular moderate exercise training, which stimulates the endogenous antioxidant system. To date, only two studies have tested the simultaneous effects of dietary antioxidant supplementation and exercise training on animal escape-response behaviour. The present study investigated the effects of dietary carotenoid supplementation and exercise training on the escape-response behaviour of Southern Corroboree frogs, *Pseudophryne corroboree*. Frogs were fed either a carotenoid-supplemented or unsupplemented diet and were exposed to repeated terrestrial escape-response trials (training) for five consecutive weeks. Carotenoid-supplemented individuals outperformed unsupplemented individuals in initial hopping speed, length of the first hop and hopping distance, however these performance measures became statistically similar by the end of the 5-week training period. Our results suggest an interaction between exercise training and dietary antioxidant supplementation. In regard to hopping speed, exercise training significantly improved the speed of unsupplemented, but not carotenoid-supplemented individuals. Our results provide some of the first evidence that exercise training improves escape performance, and that dietary antioxidants may inhibit training-induced benefits.

*Keywords:* amphibian; carotenoids; diet; escape-response; exercise; nutrition.

## **1. INTRODUCTION**

Locomotory escape-response behaviour (i.e. running, flying, swimming, and hopping) is displayed by many animals in response to threatening stimuli, such as predation (Edmunds,

1974). While effective escape-response behaviour can improve an individual's probability of survival, such behaviour is energetically costly and can place an individual's body under substantial physiological stress, which can have negative impacts on lifetime fitness. Periods of acute exercise, such as escape-response episodes, can increase an individual's metabolic rate up to 100 times, generating an overproduction of reactive oxygen species (ROS) (Larcombe et al., 2010). The result is impaired cellular redox balance and oxidative stress, which can cause damage to muscle tissue, lipids, proteins and DNA, and result in muscle atrophy, fatigue, and contractile muscle dysfunction (Powers et al., 2004, Isaksson et al., 2011). Oxidative stress can be reduced by supplementing individuals with dietary antioxidants (exogenous antioxidants) or through regular moderate exercise, which stimulates the endogenous antioxidant system (Powers et al., 2004).

Research in this field has largely focused on the effect of dietary antioxidant supplementation on exercise performance in human and laboratory mammals (Sacheck et al., 2000, Aoi et al., 2003, Urso and Clarkson, 2003, Powers et al., 2004, Shafat et al., 2004, Ryan et al., 2010). These studies have generally reported that supplementation improves performance, leading to the broad recommendation that dietary antioxidants be used as performance enhancers (reviewed by Powers et al., 2004). More recently, the antioxidant effects of carotenoids on exercise performance in non-mammalian vertebrates has gained research attention, with a growing number of studies demonstrating positive effects of dietary carotenoids on antipredatory escape-response behaviour (Blount and Matheson, 2006, Arnold et al., 2010, Larcombe et al., 2010, Silla et al., 2016). These studies have shown that carotenoids positively influence escape time, stamina, and endurance, as well as the proportion of intact DNA following escape-response trials (Blount and Matheson, 2006, Arnold et al., 2010, Silla et al., 2016).

Despite these advances, past research investigating the effects of dietary antioxidants on performance has generally only tested single episodes of escape-response (Blount and Matheson, 2006, Silla et al., 2016). The physiological stress experienced during repeat, regular escape-response events is likely to be markedly different to that experienced during a single, isolated escape-response event. Numerous studies have shown that moderate regular exercise can reduce physiological stress (Cummings, 1979, Miller and Camilliere, 1981, Oztasan et al., 2004, Radak et al., 2008). The reduction in physiological stress occurs because regular moderate exercise creates small amounts of ROS in muscle tissue, which stimulates the activation of endogenous antioxidant enzymes that occur naturally in the body (Oztasan et al., 2004, Gomez-Cabrera et al., 2008, Radak et al., 2008, Arnold et al., 2010, Larcombe et al., 2010). Exposure to regular exercise training events, and the subsequent upregulation of endogenous antioxidants, can therefore limit the oxidative stress experienced by muscle tissue (Oztasan et al., 2004). So, while studies have focused on how dietary antioxidant supplementation can improve escape-response, few have considered how exercise training might interact with dietary supplementation to influence overall escape-response behaviour.

To our knowledge, only two studies (both in the same avian model; budgerigar, *Melopsittacus undulates*), have investigated the effect of both dietary carotenoid supplementation and regular exercise training on escape-response behaviour (Arnold et al., 2010, Larcombe et al., 2010). Both studies found that exercise training improved escape-response, and that training-induced improvements in performance were enhanced in birds that also received a dietary antioxidant supplement (Arnold et al., 2010, Larcombe et al., 2010). To ascertain the generality of these findings, there is a need to understand how both exercise training and dietary antioxidant supplementation affect overall escape-response in a wider range of taxa. A recent study in the Southern Corroboree frog (*Pseudophryne corroboree*) reported that carotenoid supplementation improved exercise endurance during aquatic and

terrestrial escape-response trials (Silla et al., 2016). The present study aimed to build on these findings by simultaneously investigating the effects of carotenoid supplementation and regular exercise training on the terrestrial escape-response of *Pseudophryne corroboree*.

## **2. METHODS**

### *2.1. Study species*

*Pseudophryne corroboree* is a small (25-30 mm, snout-vent length), long-lived ( $\leq 9$  years in the wild) anuran (family Myobatrachidae) endemic to the Snowy Mountain region of New South Wales (Osborne, 1989, Osborne, 1991, Hunter et al., 2009). *P. corroboree* is a slow developing species that does not reach sexual maturity until approximately three years of age (Osborne, 1991). *P. corroboree* is relatively sedentary, and, as in other *Pseudophryne* species, locomotion is characterised by slow crawling movements. However, when threatened, frogs display escape-response behaviour by hopping short distances (Colefax, 1956a, Silla et al., 2016). The diet of this species is known to consist of algae and detritus in the larval life-stage, and insects in the adult life-stage (Osborne, 1991), all of which contain carotenoids (Lichtenthaler, 1987, Eeva et al., 2010).

### *2.2. Husbandry and nutrition*

Fertilised *P. corroboree* eggs were obtained from a captive breeding colony held at Melbourne Zoo, Australia. Eggs were transported to the University of Wollongong on July 19, 2013. Following arrival, eggs were stimulated to hatch via flooding with reverse-osmosis (R.O.) water. During development (tadpole life-stage) and adulthood (post-metamorphic life-stage), frogs were housed individually according to husbandry protocols described elsewhere (McInerney et al., 2016, Silla et al., 2016). Briefly, tadpoles were assigned to either a carotenoid-supplemented diet or an unsupplemented diet upon hatching (N = 23 frogs per

treatment). The unsupplemented tadpole diet consisted of 2 g of ground fish flakes (75:25 mixture of Sera Flora/ Sera Sans) suspended in 20 ml of R.O. water and drawn into syringes to allow for even proportionment of food among individuals. The carotenoid-supplemented diet for tadpoles consisted of the unsupplemented diet plus 0.04 g of a carotenoid mixture (Superpig; Rapashy®, CA, USA). Syringes containing food for each dietary treatment were made in large batches, frozen, and thawed immediately prior to feeding. Tadpoles received two droplets three times a week (range = 0.015 g – 0.018 g dry mass) for the first two months of the experimental period. After this time, food quantity was increased to four droplets (range = 0.03 g – 0.036 g dry mass) until the beginning of metamorphosis (forelimb emergence: Gosner stage 42). While metamorphosis was occurring (Gosner stage 42 to 46), individuals were not fed as re-absorption of the tail meets nutritional requirements during this developmental period.

Once metamorphosis was complete, the adult unsupplemented diet consisted of live *Acheta domestica* crickets twice weekly, such that food was available *ad libitum*. Forty-eight hours prior to being fed to the frogs, crickets were fed pieces of granny-smith apple. The carotenoid-supplemented diet also consisted of live crickets, though crickets were fed pieces of carrot 48 h prior to being fed to the frogs, and were dusted with 1 g of carotenoid mixture (Superpig; Rapashy®, CA, USA) immediately before being offered to frogs. Once a week, crickets in both dietary treatments were dusted with 0.2 g of calcium powder (Repti-Cal, AristoPet, Australia) to prevent calcium deficiencies. The carotenoid doses provided to frogs in the present study were based on those previously used to investigate the effects of dietary carotenoids on growth and development (Byrne and Silla, 2017), colouration (Umbers et al., 2016), escape-response behaviour (Silla et al., 2016) and foraging efficiency (McInerney et al., 2016) in the Southern Corroboree frog. Throughout the tadpole and adult life-stages, ambient temperature ranged from 10 – 20 °C and was cycled to reflect natural seasonal



changes including a hibernation period over winter. Fluorescent lighting in the room was set to simulate a normal 11.5hr/12.5hr day-night cycle experienced by *P. corroboree* in nature. Individuals were also provided with one hour of UV-B light per day (1130 – 1230 hrs) provided by a Reptisun<sup>®</sup> (Zoo Med, Germany) UV-B light bulb (36” fluorescent strip bulb) that was suspended approximately 20cm above experimental containers. UV-B light was provided to prevent UV deficiencies which can negatively impact growth and development of anurans (Lannoo, 2008).

### *2.3. Exercise training and escape performance*

Experimental trials were conducted from December 31, 2015- February 5, 2016 when individuals were approximately two years of age. The terrestrial escape performance of frogs (N = 23 per treatment) was quantified prior to training in week 1, and again after five consecutive weeks of repeat escape-response trials (training). Training was conducted every Thursday and Friday between 0900 and 1300 hrs. Individuals from each treatment were randomly assigned to a training day (either Thursday or Friday) and were given a unique identification number so that treatment was unknown to the observer at the time of experimentation. Each training day contained equal representatives from each diet treatment, and training order remained the same throughout experimental weeks to minimise temporal effects. Individuals were placed at the beginning of a plastic race track (30 cm x 4 cm and 4 cm high) and pursued by a model Alpine Copperhead snake (*Austrelaps ramsayi*), found in the same range as *P. corroboree*. Prior to the commencement of each escape-response trial, individuals were shielded from the snake by an opaque divider. Once training began, the opaque divider was removed and the snake approached the frog via an automated pulley system (Tamiya Corp., Aliso Viejo, CA, USA) powered by a 12 V 36 RPM motor (Soanar, Sydney, NSW, Australia) and 12 V DC speed controller (Plus Switch mode regulated plug pack (Powertech, Taiwan, China) to standardise attack speed. The speed of the model

predator remained constant for all trials (0.75 cm/s) (see Supplementary Movie S1). Methods used in the present study were based on those previously used to investigate escape performance in *P. corroboree* (Silla et al., 2016). During a previous study we observed that individuals remained active, with no signs of lethargy, after fleeing from the simulated predator. Based on this observation, we assumed that the training regime imposed in the present study was not exhaustive. However, because *P. corroboree* is a relatively sedentary species, the trials imposed forced individuals to move further and faster than observed during normal activity (McInerney unpublished data). For this reason, the level of training imposed was assumed to represent a moderate level of exercise. After each trial, the race track was wiped clean with ethanol and R.O. water to ensure any scent trail from previous individuals were removed. Escape-response trials in week 1 (before training), and in week 5 (after training) were video recorded using a high definition Sony Exmor R Handycam mounted to a tripod approximately 50 cm above the hopping track. Videos from these weeks were later re-watched in Windows Movie Maker software and; (1) hopping speed (cm/s), (2) length of the first hop (cm) and (3) hopping distance (cm) were quantified in response to the first tap from the model predator. When video recordings were re-watched, blinded methods were used to ensure that diet treatment was unknown to the observer during data collection and analysis. Trials were conducted at a constant temperature of 15 °C and light settings followed a 11: 13 hr light: dark cycle, including a 15 min dim-lighting period at both dawn and dusk.

#### 2.4. Statistical analysis

To test the effect of diet treatment on hopping speed, length of the first hop and hopping distance before and after the 5-week training period, we ran three separate Wilcoxon 2-sample test models. In each model, the explanatory variable was diet treatment and the response variable was either; hopping speed, length of the first hop or hopping distance. To test for within treatment effects on hopping speed, length of the first hop and hopping

distance before and after training, we ran three separate Wilcoxon signed rank tests for each treatment. In each model, the explanatory variable was training week and the response variable was the performance variable. Also, because performance in frogs can be influenced by body size, regression analyses were used to examine the association between body mass and each performance measure. Within each diet treatment, body mass did not have a significant influence on any of the performance measures examined. As such, body mass was not included as a covariate in any of the subsequent statistical analyses. All analyses were conducted using JMP Pro 11 (SAS Institute, Cary, NC, USA), with significance levels set to  $P < 0.05$ .

### *2.5 Ethical Note*

All procedures described in this study were conducted following evaluation and approval of the University of Wollongong's Animal Ethics Committee (approved number AE 13/13). All experimental procedures followed ASAB/ABS guidelines

## **3. RESULTS**

### *3.1. Hopping speed (cm/sec)*

Carotenoid supplemented individuals performed significantly better than unsupplemented individuals in week one prior to training (Wilcoxon test,  $N = 23$ ,  $Z = -2.32$ ,  $P = 0.02$ ; Fig. 1a). After the 5-week training period, the performance of frogs in each treatment was statistically similar (Wilcoxon test,  $N = 23$ ,  $Z = 0.71$ ,  $P = 0.48$ ; Fig. 1a). Between weeks one and five the hopping speed of frogs in the unsupplemented dietary treatment improved significantly after training (Wilcoxon signed-rank test,  $N = 23$ ,  $T_{22} = 2.50$ ,  $P = 0.01$ ), while the hopping speed of carotenoid-supplemented frogs did not improve significantly after training (Wilcoxon signed-rank test,  $N = 23$ ,  $T_{22} = -0.93$ ,  $P = 0.46$ ).

### 3.2. Length of initial hop (cm)

Carotenoid supplemented individuals performed significantly better than unsupplemented individuals in week one, prior to training (Wilcoxon test,  $N = 23$ ,  $Z = -2.26$ ,  $P = 0.02$ ; Fig. 1b). After the 5-week training period, the performance of frogs in each treatment was statistically similar (Wilcoxon test,  $N = 23$ ,  $Z = -0.73$ ,  $P = 0.46$ ; Fig. 1b). Between weeks one and five there was no significant change in the length of the initial hop exhibited by frogs in either the unsupplemented dietary treatment (Wilcoxon signed-rank test,  $N = 23$ ,  $T_{22} = 1.32$ ,  $P = 0.19$ ), or the carotenoid-supplemented dietary treatment (Wilcoxon signed-rank test,  $N = 23$ ,  $T_{22} = -0.67$ ,  $P = 0.59$ ).

### 3.3. Hopping distance (cm)

Carotenoid supplemented individuals performed significantly better than unsupplemented individuals in training week one (Wilcoxon test;  $N = 23$ ,  $Z = -2.05$ ,  $P = 0.03$ ; Fig. 1c). After the 5-week training period, the performance of frogs in each treatment was statistically similar (Wilcoxon test,  $N = 23$ ,  $Z = -0.09$ ,  $P = 0.93$ ; Fig. 1c). Between weeks one and five there was no significant change in the hopping distance of frogs in either the unsupplemented dietary treatment (Wilcoxon signed-rank test,  $N = 23$ ,  $T_{22} = 1.47$ ,  $P = 0.14$ ) or the carotenoid-supplemented dietary treatment (Wilcoxon signed-rank test,  $N = 23$ ,  $T_{22} = 0.09$ ,  $P = 0.86$ ).

## 4. DISCUSSION

Despite a growing number of studies investigating the effects of dietary antioxidant supplementation on animal escape-response behaviour, few have considered the combined effects of dietary supplementation and exercise training on escape performance. The present study tested the effects of exercise training and dietary carotenoid supplementation on escape

performance in the Southern Corroboree frog, *Pseudophryne corroboree*. Results show that carotenoid-supplemented individuals outperformed unsupplemented individuals in initial escape-response trials (prior to exercise training). These results are consistent with a previous study in *P. corroboree*, which reported improved exercise endurance during aquatic and terrestrial escape-response trials in individuals receiving long-term dietary carotenoid supplementation (Silla et al., 2016). Following five weeks of regular training, however, the performance of carotenoid-supplemented frogs remained relatively constant, while the performance of unsupplemented frogs tended to improve. As such, at the end of the training period the performance of frogs became statistically similar across all three response variables (initial hopping speed, length of first hop and hopping distance).

After the five-week training period, the performance of unsupplemented individuals did not significantly change in regard to length of first hop or hopping distance, but showed a statistically significant improvement in hopping speed, with average speeds almost doubling between trial week one and five. The observed increase in hopping speed may have been a result of the upregulation of endogenous antioxidants improving overall antioxidant capacity. However, this conclusion will remain speculative until levels of ROS and endogenous antioxidants are quantified following training episodes to demonstrate a causal relationship between carotenoid intake and improved performance. Nevertheless, moderate levels of ROS, generated during regular low to moderate intensity exercise training, have previously been shown to stimulate the activity of the endogenous antioxidant system (Oztasan et al., 2004, Gomez-Cabrera et al., 2008). Once activated, endogenous antioxidants such as superoxide dismutase, peroxidase, and glutathione, bind to and stabilise ROS to reduce oxidative stress (Oztasan et al., 2004, Powers et al., 2004, Gomez-Cabrera et al., 2008, Peternelj and Coombes, 2011). Training-induced reductions in oxidative stress may improve overall performance by limiting DNA damage and aiding with muscle recovery. For example a study

in the African clawed frog, *Xenopus laevis*, reported that frogs exposed to daily swim training exhibited improved sprint performance, such that the time taken to swim a distance of 1.5 m decreased by 30% over an 18 day training period (Miller and Camilliere, 1981). Trained frogs also exhibited significantly lower concentrations of lactate in muscle tissue compared to untrained controls, suggesting better muscle recovery in trained individuals (Miller and Camilliere, 1981).

While trained frogs in the present study showed improved terrestrial escape performance, we did not see the same training-induced improvement in performance in carotenoid-supplemented individuals. It has previously been suggested that dietary antioxidant supplementation may interfere with the benefits of exercise training by limiting the ability of cells to adapt to increases in ROS, and inhibiting the upregulation of endogenous antioxidants (Oztasan et al., 2004, Gomez-Cabrera et al., 2008). Our results are consistent with a growing number of studies in mammalian models that highlight the benefits of exercise training on improving antioxidant capacity and performance, while reporting that dietary antioxidant supplementation during training limits these performance improvements (reviewed in Peternelj and Coombes, 2011).

To date, only two studies have investigated the impact of exercise training and dietary antioxidant supplementation on the antipredatory escape-performance of animals (Arnold et al., 2010, Larcombe et al., 2010). Research on budgerigars has shown that exercise training improves escape-response, and that these positive effects are further enhanced in birds that also received a dietary antioxidant supplement (Arnold et al., 2010, Larcombe et al., 2010). One possible explanation for why the results of the present study are in contrast with those of the studies on budgerigars, is the difference in exercise intensity imposed. Although regular moderate exercise results in improved performance by allowing the body to become more resistant to oxidative challenges, the impact of acute exercise is markedly different (Peternelj

and Coombes, 2011). Vigorous or exhaustive exercise can lead to extreme increases in ROS production, which can overwhelm and exceed the capacity of the endogenous antioxidant system (Peternelj and Coombes, 2011), and the benefits of dietary supplements are more likely to be observed. The two studies assessing the effect of training and antioxidant supplementation on escape performance in budgerigars exposed birds to six repeat take-off flights, designed to be exhaustive, over eight to nine weeks of consecutive training (Arnold et al., 2010, Larcombe et al., 2010). As a result, the escape-response training that budgerigars experienced was of a much higher intensity than the escape-response training imposed in the present study. Frogs in the present study were only subjected to a single, non-exhaustive, escape-response trial once every seven days for five consecutive weeks of training, so the exercise training imposed was both of lower intensity and occurred over a shorter time period. Consequently, ROS generation may have been lower by comparison and the antioxidant requirements of frogs may have been met through the upregulation of endogenous antioxidants without the additional benefits of dietary antioxidant supplementation.

Furthermore, it is important to note that the antioxidant requirements of *P. corroboree* may differ to other taxa and, in particular other anurans. *Pseudophryne corroboree* is a relatively sedentary species and individuals only crawl or take short hops when threatened (Colefax, 1956b, Silla et al., 2016). We might expect more mobile anuran species to have higher metabolic rates and therefore higher antioxidant requirements. In order to ascertain whether ROS generation and antioxidant capacity differs depending on the intensity and frequency of escape-response trials imposed, future studies would benefit from quantifying ROS production and oxidative stress in response to training and dietary supplementation.

Overall, results from our study provide preliminary evidence for an interaction between exercise training and dietary antioxidant-supplementation on escape-performance in the Southern Corroboree frog. In regard to hopping speed, our results show that exercise training

improved the speed of unsupplemented, but not carotenoid-supplemented individuals. Data provide a first-step towards better understanding the influence of endogenous and exogenous sources of antioxidants on amphibian anti-predatory escape-response behaviour. The benefits of exogenous antioxidant supplements on escape-response behaviour are likely to be taxa specific and linked to the intensity of exercise imposed. Future research would benefit from investigating the influence of exercise training and antioxidant supplementation on escape performance in a wider range of taxa with different energetic costs of escape-response behaviour. Future studies would also benefit from quantifying ROS production and oxidative stress in response to training and dietary supplementation. Gaining this information would advance our understanding of the influence of dietary antioxidants and exercise training on individual escape performance.

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## Figures

**Fig. 1:** Effect of carotenoid supplementation and exercise training on (a) hopping speed (cm/s), (b) length of the first hop (cm) and (c) hopping distance (cm) in *P. corroboree* (N = 23 frogs per treatment). Data are untransformed means  $\pm$ SEM.

*See file 'Fig. 1 TIFF'*

## Supplementary Movies

**S1:** Example of the training sessions used to quantify escape-performance of Southern Corroboree frogs.

*See file 'Supplementary Movie S1'*

