Winter January 24, 2018

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Comparative Biochemistry and Physiology, Part A 218 (2018) 56–62

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Maternal allocation of carotenoids to eggs in an *Anolis* lizard

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ARTICLE INFO

Keywords:
Antioxidants
Carotenoids
Maternal allocation
Paternal effects
Pro-oxidants

ABSTRACT

The maternal allocation of carotenoids to eggs has been widely documented and manipulated. However, it is often assumed that the sole adaptive value of this allocation is to increase offspring fitness. Because carotenoids can be pro-oxidants or antioxidants depending on their concentrations and their chemical environment (i.e. presence of other antioxidants), dams may need to dispose of excess carotenoids upon depletion of other antioxidants to prevent oxidative damage. Additionally, the amount of carotenoids deposited in eggs may be dependent on male traits such as quality and coloration. We evaluated these two non-mutually exclusive hypotheses for carotenoid allocation to eggs and assessed paternal effects by supplementing male and female brown anole lizards, *Anolis sagrei*, with dietary carotenoids or with a combination of carotenoids and vitamin C. We found significant differences in the antioxidant capacities of fertilized and unfertilized eggs produced by female lizards, but the treatment did not affect the antioxidant capacity or carotenoid content of eggs. However, the carotenoid concentration of unfertilized eggs from carotenoid-supplemented females was significantly higher than eggs from the control group. Male coloration and body size did not affect the antioxidant capacity or carotenoid content of the eggs. Carotenoids may be allocated to unfertilized eggs to offset oxidative damage to the dam, with a neutral effect on offspring, rather than to solely provide antioxidant benefits to offspring as has been widely assumed.

1. Introduction

Cellular processes impact life history, behavior, and fitness. Reactive oxygen species (ROS) are produced as a natural byproduct of most mitochondrial actions (Balaban et al., 2005). If unmatched by antioxidant mechanisms, ROS can lead to a number of harmful and consequential effects collectively termed oxidative stress (Catoni et al., 2008; Monaghan et al., 2009). The antioxidant system is made up of a complex network of endogenous and dietary molecules that mitigate oxidative stress by removing or stopping unstable ROS and their chain reactions (Vertuani et al., 2004).

Many pigments have antioxidant capabilities that may affect an animal's immune system and oxidative status (McGraw, 2005). Carotenoids create red, yellow, and orange colors and have been well-studied because of their signaling functions (Svensson and Wong, 2011) but can also have potent oxidant activity (Palozza, 1998; El-Agamey et al., 2004). Carotenoids cannot be created de novo by any vertebrates and so must be obtained from the animal's diet or from maternal reserves during development, which often makes them energetically costly to obtain and metabolize (Goodwin, 1984; Olson and Owens, 1998). The maternal allocation of carotenoids to eggs has been most frequently studied in birds and fish (e.g. Craik, 1985; Verakunpiriya et al., 1997; Blount et al., 2003a) and most studies assume dams allocate carotenoids to eggs to provide developing offspring benefits such as increased immune function and antioxidant effects (Haq et al., 1996; Romano et al., 2008; Svensson and Wong, 2011). Because embryonic development is a time of high risk of oxidative damage, the antioxidant network is especially important during this stage (Costantini and Møller, 2008; Costantini et al., 2008). Offspring from dams supplemented with carotenoids have higher levels of circulating carotenoids (Surai et al., 2003; Ewen et al., 2006; Brown et al., 2014) and in some cases mature to have increased fecundity (Verakunpiriya et al., 1997), decreased susceptibility to oxidative damage (Surai et al., 2003; McGraw et al., 2005a; Blount et al., 2012; Casagrande et al., 2014), and increased survival and growth (George et al., 2001; McGraw et al., 2005a; Brown et al., 2014).

Molecules with similar antioxidant functions do not necessarily share common structural characteristics; in fact, many can be potent antioxidants *or* pro-oxidants depending on the molecular context (Vertuani et al., 2004). Carotenoids, though often studied as honest signals that convey information regarding immune function (Blount et al., 2003b), social status (Pryke et al., 2002), or oxidative stress status (Casagrande et al., 2014), must be reduced by other antioxidants, such as vitamin C or E, to have antioxidant effects (Catoni et al., 2008).

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https://doi.org/10.1016/j.cbpa.2018.01.014
Received 10 November 2017; Received in revised form 23 January 2018; Accepted 24 January 2018
Available online 02 February 2018
1095-6433/

These effects include not only the direct scavenging and quenching of free radicals, but also the promotion and activation of other antioxidant systems (Krinsky, 1989; Stahl and Sies, 2003; El-Agamey et al., 2004; Ben-dor et al., 2005). In the absence of other antioxidants, or at a concentration that overwhelms other antioxidants, carotenoids negatively impact the oxidative stress status of an organism (Catoni et al., 2008; Bouayed and Bohn, 2010). For instance, Giraudeau et al. (2013) found a higher level of oxidative damage in finches supplemented with carotenoids than in those supplemented with carotenoids and vitamin E. Since maternal antioxidants such as vitamin C and E become depleted during egg development, maternal carotenoids may function as pro-oxidants during ovulation. Thus, an alternative and non-mutually exclusive explanation for maternal deposition of carotenoids to eggs is that it serves to reduce oxidative damage to dams while having a neutral or positive impact on offspring health (because other antioxidants are present in the eggs).

Females may also adjust their carotenoid investment to eggs based on male traits. Paternal nutrition, paternal environmental exposure, and paternal behaviors have been shown to have trans-generational effects in multiple systems and it is well-known that females may adjust their investment to offspring based on the quality of their mate (Curley et al., 2011). For example, Bolund et al. (2009) found that zebra finches mated to low quality males deposited fewer carotenoids into their eggs. However, Saino et al. (2002) found that barn swallows mated to experimentally manipulated low quality males deposited more carotenoids into their eggs than controls. Thus, the amount of carotenoids allocated to eggs may vary based on traits of the sire, and the allocation strategy used by females may differ by system.

The brown anole, *Anolis sagrei*, is a small oviparous lizard that lays a single egg approximately every ten days during a protracted breeding season (Andrews, 1985). While one egg is ovulated, the next egg is simultaneously yolked on the contralateral follicle (Lee et al., 1989). Following ovulation, eggs are fertilized in the oviduct using sperm that can be stored for several months (Calsbeek et al., 2007). Fertilization induces shelling of the egg. Infertile eggs are laid as unshelled “slugs”, which can be readily identified by their yellow coloration. Anoles are ideal organisms with which to investigate maternal allocation since they typically lay single eggs rather than multi-egg clutches. Because carotenoid content can be affected by laying order in organisms with multi-egg clutches, such as birds (Saino et al., 2002; Romano et al., 2008; Safran et al., 2008), studying maternal allocation in an organism that lays single eggs is necessary to remove this effect.

We supplemented the diets of female *A. sagrei* with carotenoids or with carotenoids and another antioxidant (vitamin C) to test hypotheses concerning the maternal allocation of carotenoids to egg yolk. Vitamin C has been shown to interact synergistically with carotenoids, mostly via molecular recycling, to increase antioxidant activity (Böhm et al., 1998; Palozza, 1998 and references therein; Catoni et al., 2008). We predicted that if carotenoids are allocated to eggs primarily as a method of increasing offspring fitness, then the antioxidant capacity and carotenoid content of eggs should increase in both treatment groups relative to the control because the oxidant activity of carotenoids do not impact their allocation by dams. By contrast, if carotenoids are allocated to eggs primarily as a method of improving maternal oxidative stress status (OSS), the carotenoid content of eggs in the carotenoid group should be higher than in the carotenoid + vitamin C and control groups, and the antioxidant capacity of the eggs may show a similar pattern. This is because carotenoids may have pro-oxidant activity in the carotenoid group but not in the carotenoid + vitamin C group. We also measured the skin coloration of dams and sires and the body size of sires to determine if egg characteristics are affected by parental traits.

2. Material and methods

2.1. Care and dietary supplementation

We obtained wild-caught adult *A. sagrei* from Big Apple Pet Supply (Orlando, FL, USA). We measured the snout to vent length (SVL) of all individuals to the nearest millimeter. Lizards were housed in a 25–26 °C room in separate ten-gallon terraria. Each terrarium contained a potted plant for perching and as a retreat site, but access to soil was blocked to prevent use as an oviposition site. Artificial grass substrate and a shallow petri dish filled with soil were provided to facilitate egg detection and collection. All tanks were illuminated by a UVB bulb and a 40 W heat lamp on an automated 12:12 cycle. Female lizards were randomly assigned to one of three dietary treatment groups: control, carotenoid, or antioxidant (carotenoid + vitamin C). Male lizards and control group females received three to four large crickets every three days while the carotenoid group received the same number of crickets dusted with commercial xanthophylls (7 g/lb, Oro-Glo Feed, Kemin Industries, Winterset, IA, USA). The antioxidant treatment group received three to four crickets dusted with a 1:1 mass of carotenoids and vitamin C in powder form (Ascorbic acid, Sigma-Aldrich, St. Louis, MO, USA). Crickets were dusted with supplemental powders by collecting them in paper cups and gently shaking the cup to coat each cricket. Diet treatments continued for twelve weeks. All work was approved by the Dartmouth College Institutional Animal Care and Use Committee (Protocol #cals.rg.1) and followed the guidelines outlined by international organizations.

2.2. Mating

After four weeks of dietary supplementation, females were allowed to mate with a randomly chosen male. Males were removed following copulation, and female enclosures were thereafter checked daily for eggs. Females were re-mated with the same male if they had not laid a fertilized egg after four weeks. Both shelled (fertilized) and unshelled eggs (unfertilized “slugs”) were weighed and frozen at −5 °C upon recovery.

2.3. Antioxidant capacity

The total antioxidant capacity of each egg was measured using a copper-reducing colorimetric assay kit (OxiSelect, TAC Assay, Cell BioLabs, Inc., San Diego, CA, USA). This assay provides a measurement of the capacity of biomolecules within a sample to undergo a single electron transfer and thus represents a measure of the organism's reductive capacity. Briefly, eggs were homogenized in diluted methanol and uric acid standards were prepared. Unshelled eggs were homogenized in full whereas shelled eggs were removed from the shell and the contents homogenized as completely as possible. After homogenization, a copper ion reagent was added to each sample or standard and prepared in duplicate within a microplate to trigger a colorimetric reaction. The absorbance spectrum of each well was measured at 490 nm (maximal absorption for copper reductants) using a microplate reader (BioRad xMark, Hercules, CA, USA). The net absorbance was calculated by subtracting the baseline measurement from the final measurement and converting to μm Copper Reducing Equivalents (CRE) by multiplying the value by 2189 μm Cu/mM, where a higher CRE is equivalent to a higher antioxidant capacity (OxiSelect™ Product Manual).

2.4. Carotenoid content

We estimated the μg/g carotenoid concentration and total carotenoid content of each egg by extracting carotenoids following McGraw et al. (2005b) but substituting methanol for ethanol. Though methanol is less effective at extracting non-polar carotenoids, we added

hexane and methyl *tert*-butyl ether to ensure successful extraction of polar and non-polar carotenoids (Kevin McGraw, pers. comm.). We used two measures for quantifying carotenoids from absorbance spectra because it is unclear whether concentration or total carotenoid content are likely to be more biologically relevant in *A. sagrei* (and many other systems; Safran et al., 2008). For both the antioxidant capacity and the carotenoid content analyses, the authors were blind to which treatment eggs belonged.

2.5. Integument coloration

Although pterins are likely partially responsible for body coloration in *A. sagrei*, there is evidence that carotenoids often co-occur with pterins in *Anolis* (Steffen and McGraw, 2009; Alfonso et al., 2013), and preliminary analyses suggest that carotenoids are more prevalent than pterins in *A. sagrei* dorsum skin (Erritouni, unpublished data). Regardless of its composition, integumentary coloration may be significantly affected by antioxidant supplementation if pigments no longer have to be diverted for oxidant activity (Weiss et al., 2011). We measured the body coloration of female lizards at the beginning and end of the experiment to determine if the dietary treatments had an impact on coloration. Additionally, we measured body coloration of females and males at the time of mating to determine if dam and sire coloration predict the carotenoid concentration, carotenoid content, and antioxidant capacity of eggs. Reflectance spectra were collected using an Ocean Optics Jaz spectrometer with a bifurcated full-spectrum light source probe fitted with a custom 45° angle cover and placed on the "reddest" portion of the skin on the side of the body. Reflectance spectra were collated into 1 nm bins and negative values (due to noise) were zeroed. Brightness was measured by calculating the integral of the spectrum. The relative carotenoid reflectance (hereafter called the carotenoid chroma) was calculated for each lizard as $[(R_{450nm:700nm})/R_{300:700nm}]$ which represents the portion of the reflectance attributable to the wavelengths around the peak absorbance of carotenoids.

2.6. Statistics

We used a linear mixed model with dam number as a random effect and egg nested within dam number to test for a significant difference in antioxidant capacity between fertilized and unfertilized eggs. We also used linear mixed models to determine how well the treatment, length of time on the treatment at the time of egg-laying, and their interaction predicted the variation in antioxidant capacity, carotenoid content, and the weight of eggs. We used Akaike's corrected information criterion (AICc) to determine if models without interactions were a better fit and report only the results from the model with the lowest AICc score. We included dams as a random effect since some individuals laid multiple eggs. Before performing the linear mixed models, we checked for homogeneity of regression slopes. We used the Satterthwaite approximations to estimate degrees of freedom since our data were unbalanced. We used ANOVA to determine if treatment affected the total number of eggs or total proportion of fertilized eggs laid by each female. We used a generalized linear model with a logit link function to determine if there was a difference in survival between treatment groups. We also used an ANOVA to test if treatment predicted the change in carotenoid chroma or brightness in dams over the course of the experiment. Finally, we constructed linear models to determine if dam carotenoid chroma, sire carotenoid chroma, or sire body size affected the average antioxidant capacity, carotenoid concentration, or weight of eggs per female. All analyses were performed in R Statistical Programming Language (R Core Team, 2016).

3. Results

Fertilized eggs had a significantly higher mean antioxidant capacity than unfertilized eggs ($t = 0.866$, $df = 78$, $P < 0.0001$;

Table 1
Fixed effects for linear mixed models of the antioxidant capacity of fertilized and unfertilized *Anolis sagrei* eggs. Unfertilized egg data are presented in parentheses.

Fixed effects	Estimate	Error	df	t	*P*
Intercept	193.85 (−890.14)	642.47 (359.03)	26.18 (38.95)	0.30 (−2.48)	0.765 (0.0176)
Days on trmt	−1.39 (3.91)	6.10 (3.92)	26.18 (38.95)	−0.23 (0.99)	0.822 (0.3252)
Control	−648.95 (−628.21)	773.16 (587.60)	26.18 (38.95)	−0.839 (−1.07)	0.409 (0.2916)
Antioxidant trmt	−4.48 (−28.97)	976.42 (535.98)	26.18 (38.95)	−0.01 (−0.05)	0.99 (0.9572)
Days × control	8.67 (8.36)	8.11 (6.70)	26.18 (38.95)	1.07 (1.25)	0.295 (0.2195)
Days × ao trmt	0.33 (2.31)	8.86 (5.96)	26.18(38.95)	0.04 (0.388)	0.970 (0.7003)

Random effects were included in the model as described in the text, but explained nearly zero of the variance. AICc weight for fertilized egg model = 0.965. AICc weight for unfertilized egg model = 1.
Asterisks denote significance at a $p < .05$ level.

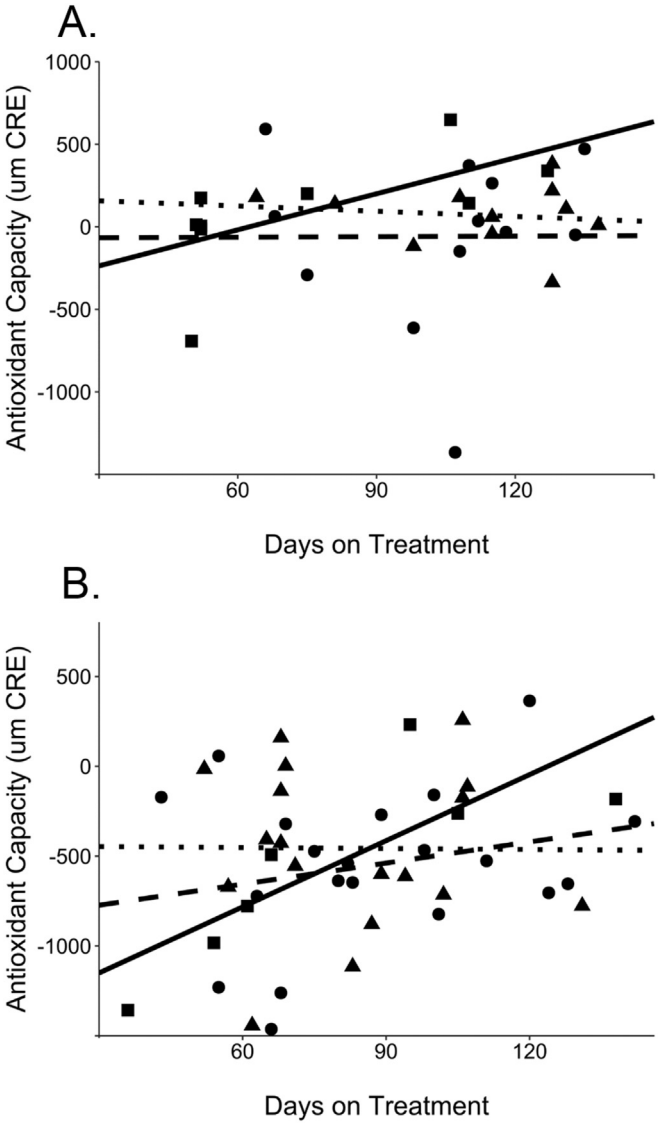

Fig. 1. The relationship between the days on the treatment at the time that *Anolis* laid an egg and the antioxidant capacity of the egg. A) Fertilized eggs. B) Unfertilized eggs. Squares and solid lines represent the control group, circles and dashes represent the carotenoid treatment, and triangles and dots represent the antioxidant treatment.

Table 2
Fixed effects for a linear mixed model of the carotenoid concentration of unfertilized *Anolis sagrei* eggs.

Fixed effects	Estimate	Error	df	t	*P*
Intercept	139.30	93.86	36.89	1.48	0.146
Days on treatment	1.14	0.97	36.89	1.17	0.249
Treatment	−115.66	51.97	36.89	−2.23	0.032*

Random effects were included in the model as described in the text, but explained nearly zero of the variance. AICc weight = 0.659.

Table 3
Fixed effects for a linear mixed model of the total carotenoid content of unfertilized *Anolis sagrei* eggs.

Fixed effects	Estimate	Error	df	t	*P*
Intercept	9.24	5.10	30	1.89	0.08
Days on treatment	0.02	0.05	30	0.44	0.66
Control	−2.97	3.04	30	−0.978	0.34

Random effects were included in the model as described in the text, but explained nearly zero of the variance. AICc weight = 1.

Table 4
Fixed effects for linear mixed models of the weight of fertilized and unfertilized *Anolis sagrei* eggs. Unfertilized egg data are presented in parentheses.

Fixed effects	Estimate	Error	df	t	*P*
Intercept	0.01 (0.05)	0.04 (< 0.01)	9.31 (24.02)	0.22 (8.14)	0.824 (< 0.001)
Days on treatment	< 0.01 (< 0.01)	< 0.01 (< 0.01)	11.78 (44.93)	1.96 (−0.31)	0.075 (0.760)
Control	< 0.01 (< 0.01)	0.02 (< 0.01)	6.43 (10.77)	−0.17 (0.25)	0.874 (0.805)
Antioxidant treatment	< 0.01 (< 0.01)	0.03 (< 0.01)	6.58 (10.28)	−0.05 (−0.05)	0.959 (0.958)

Random effects were included in the model as described in the text, but explained nearly zero of the variance. AICc weights for both models = 1.

$\bar{x}_{\text{fertilized}}$ = 68.49 μm CRE, SE = 77.57; $\bar{x}_{\text{unfertilized}}$ = −481.46 μm CRE, SE = 71.99). We found no significant effect of treatment or treatment duration at the time of egg-laying on antioxidant capacity for either fertilized eggs or unfertilized eggs (Table 1, Fig. 1).

All eggs from the antioxidant treatment group had carotenoid levels below detection limits (all = 0 μg/g) so were removed from this analysis but we discuss this result below. We found a significant effect of treatment (control and carotenoid only) but not treatment duration on the carotenoid concentration of unfertilized eggs (Table 2, Fig. 2; $\bar{x}_{\text{control}}$ = 137.62 μg/g, SE = 26.94, $\bar{x}_{\text{carotenoid}}$ = 242.90 μg/g, SE = 36.30) but not the total carotenoid amount (Table 3) of unfertilized eggs. Finally, we found no significant effect of treatment or treatment duration on the mass of unfertilized or fertilized eggs (Table 4, Fig. 3).

Treatment had no effect on the date of initial oviposition (ANOVA $F_{2,31}$ = 1.17, P = 0.32), on the total number of eggs laid ($F_{2,31}$ = 0.717, P = 0.496), nor on the proportion of fertilized eggs ($F_{2,31}$ = 0.113, P = 0.893) laid by females who laid eggs. There were no differences in survival among treatment groups ($\bar{x}_{\text{control}}$ = 0.47, SE = 0.13; $\bar{x}_{\text{carotenoid}}$ = 0.6, SE = 0.13; $\bar{x}_{\text{antioxidant}}$ = 0.73, SE = 0.12). We found no significant effect of treatment on the change in dam carotenoid chroma (ANOVA $F_{2,24}$ = 0.009, P = 0.991) or brightness (ANOVA $F_{2,24}$ = 0.476, P = 0.627). Dam carotenoid chroma, sire carotenoid chroma, and sire body size were not correlated with the antioxidant capacity, carotenoid concentration, carotenoid content, or weight of unfertilized or fertilized eggs (P > 0.05 for all).

4. Discussion

We did not find an effect of carotenoid supplementation, either alone or with vitamin C, on the antioxidant capacity or carotenoid content of eggs. However, we found a significant difference in the carotenoid concentrations of unfertilized eggs from the control group and from the carotenoid supplemented group, suggesting that with the dietary addition of carotenoids, females deposit carotenoids into unfertilized eggs to get rid of them (Fig. 2). The levels of carotenoids in all eggs from the antioxidant treatment group were below the detection limits of the absorbance spectrometer. Our carotenoid content analyses were destructive, so we were unable to further analyze eggs in the antioxidant treatment to determine if the lack of carotenoids detected was due to technical errors. Since the original carotenoid content analyses were conducted blindly with respect to treatment, we suggest that the inability of the test to detect any carotenoids present in the eggs from the antioxidant treatment is not due to methodological error, but rather that the metabolism of carotenoids in the presence of vitamin C is more biochemically complicated than expected. For example, carotenoids and vitamin C may compete for binding or transport sites.

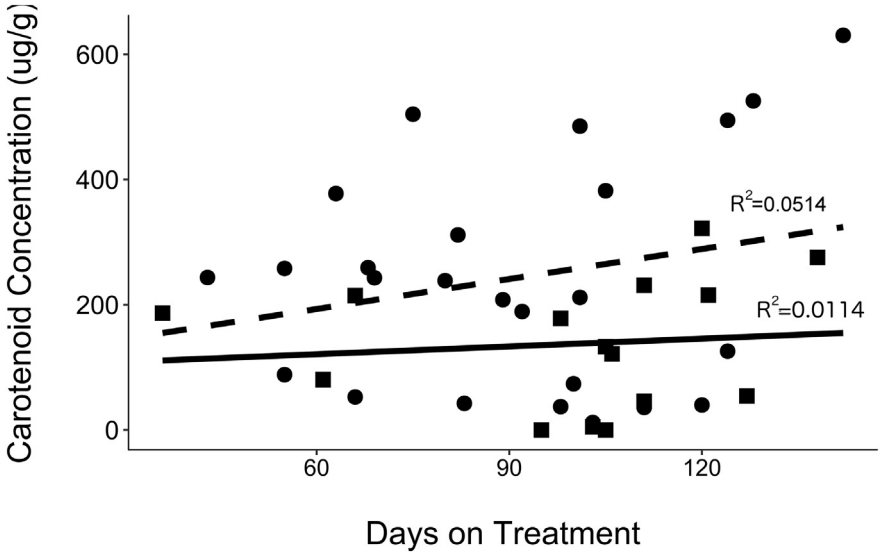

Fig. 2. The carotenoid concentration of unfertilized *Anolis* eggs increases with the time on the treatment in the control and carotenoid treatment groups. Solid line represents the control and dashed line represents the carotenoid treatment.

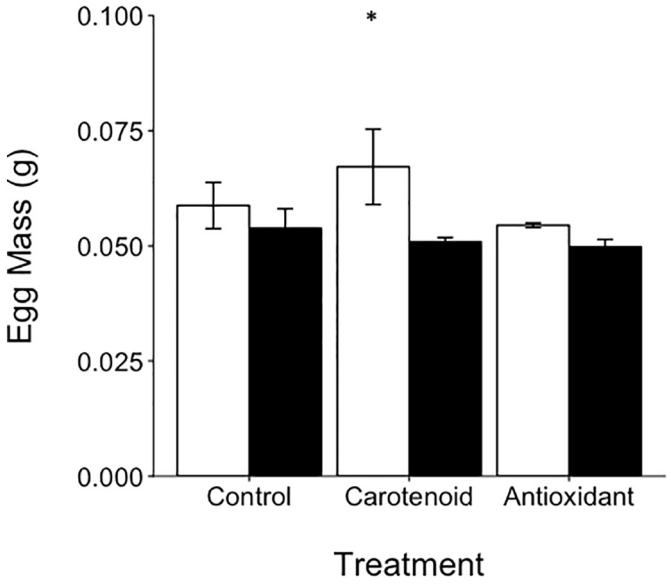

Fig. 3. Egg weight did not significantly differ among treatment groups. White bars represent fertilized (shelled) eggs while black bars represent unfertilized eggs. Error bars show ± 1 S.E. and an outlier in the carotenoid group (egg mass = 0.218) is denoted with an asterisk.

Alternatively, this pattern could be explained if carotenoids are metabolized differently by dams depending on the availability of other antioxidants. Similarly, Blount et al. (2002) found that after supplementing a fixed carotenoid cocktail to female blackbacked gulls (*Larus fuscus*), their egg yolks contained a different ratio of carotenoid types between treatments, and they proposed that this is because of differential metabolism and/or physiological discrimination of carotenoid types by females. Antioxidant activity can vary by carotenoid type (Blount et al., 2002), so females may alter or discriminate between types to maximize benefits to offspring while regulating their own oxidative state. Grether et al. (2008) proposed a similar explanation after finding that female guppies likely metabolize carotenoids before expressing them in their skin, but seem to not alter the carotenoids obtained from the diet before depositing them in eggs. Additionally, work by Cantarero and Alonso-Alvarez (2017) has recently shown that antioxidants within mitochondrial membranes can directly mediate the metabolization of carotenoids in birds. These results accentuate the importance of profiling carotenoids in future studies of anoles and other organisms to better infer the amount of energy necessary to metabolize, deposit, or express carotenoids. More complex analyses (e.g. high-performance liquid chromatography) may have better success at identifying the forms and quantity of carotenoids present, if any.

When supplemented with only carotenoids, females increase the carotenoid concentration of their unfertilized eggs relative to the eggs laid by control females, but this does not appear to be true for females supplemented with carotenoids and vitamin C together, regardless of the mechanism by which carotenoids are left out of eggs in this group (Table 2). This is evidence in support of the hypothesis that carotenoid deposition to eggs serves to reduce oxidative damage to dams while having a neutral or positive impact on offspring health. Because there is no subsequent increase in the antioxidant activity of eggs of carotenoid-supplemented females, additional carotenoids appear to have a neutral impact on early offspring health. However, further work needs to be done to verify that carotenoids are truly absent from the eggs of antioxidant-supplemented females in order to provide more support for this hypothesis, and an understanding of the mechanism of maternal allocation would also be beneficial (e.g. active or passive transport of nutrients to eggs). Though we did not measure excrement, and it is possible that some carotenoids were disposed of through that mechanism, it is interesting that dietary carotenoids were excreted into unfertilized eggs at all because this suggests that carotenoids were uptaken from the diet rather than just passively disposed of through the digestive system.

It is unclear why carotenoid concentration, but not carotenoid content, was affected by carotenoid supplementation. Again, understanding the mechanism of carotenoid deposition (active vs. passive) may shed light on this issue. Other studies have also found different relationships between egg contents based on whether concentrations or total amounts have been used and have stated that continuing to measure both variables is essential for comparative studies and advancing the understanding of parental allocation (Safran et al., 2008; Blount et al., 2012).

Fertilized eggs had a higher antioxidant capacity than unfertilized eggs, in contrast to what has been found in another lizard, *Sceloporus virgatus* (Weiss et al., 2011). Because anoles do not shell their eggs until after fertilization, the difference in antioxidant capacities suggests that females may have the ability to allocate additional nutrients and antioxidants to eggs before they are completely shelled (Weiss et al., 2011). Alternatively, eggs with more antioxidants may be more likely to successfully become fertilized, though little work has been done to address this possibility in any system. Additionally, although eggs were collected within one day of deposition, embryo development could have already contributed to antioxidant levels by the time of laying.

Assays of total antioxidant capacity are able to measure levels of circulating antioxidants but not enzymatic components (Monaghan et al., 2009). Thus, eggs with a lower measured antioxidant capacity may not necessarily be disadvantaged relative to eggs with a higher measured antioxidant capacity since this difference could be counterbalanced with enzymatic antioxidants. Nonetheless, many studies that manipulate maternal dietary antioxidants find both an increase in yolk antioxidant capacity and an increase in offspring survival, presumably because of an early antioxidant advantage during development (Surai et al., 2003; McGraw et al., 2005a; Brown et al., 2014).

In some species, egg size is correlated with survival and performance (Sinervo, 1990; Wagner and Williams, 2007). We found no significant differences in egg size among treatments or treatment durations (Table 4). Though fertilized eggs from the carotenoid group had greater mass, this effect was not significant when multiple eggs from the same females were controlled for (Fig. 3, Table 4).

We found no effect of carotenoid supplementation on dam dorsal skin coloration. In birds, females supplemented with carotenoids were both more colorful and provided more antioxidants to their eggs (McGraw et al., 2005a). However, a study on *Ctenophorus* lizards found no effect of carotenoid supplementation on skin coloration or circulating reactive oxygen species (Olsson et al., 2008) and carotenoid supplementation also did not impact the skin coloration of lacertid lizards (Fitze et al., 2009). Similarly, Steffen et al. (2010) found no difference in *Anolis* dewlap coloration between groups supplemented and deprived of carotenoids. Our results support their conclusion that *Anolis* carotenoid coloration is not sensitive to dietary input in adults. An interesting expansion to our study would involve raising the offspring of treated females to determine if there is an effect of maternal carotenoid supplementation on offspring integument and dewlap coloration.

Similarly, raising the offspring of carotenoid-supplemented females may reveal fitness effects not detected within the egg. For instance, cichlids supplemented with carotenoids showed no difference in coloration or in the carotenoid content of their eggs relative to control fish, but their offspring showed increased growth and survival (Brown et al., 2014). Brown et al. (2014) suggest that this discrepancy may be explained by the conversion of carotenoids to retinols that takes place in fish eggs and would be missed by the carotenoid-detection techniques. However, no similar conversion is known to take place during *Anolis* ovulation.

Though there is a growing body of literature on paternal effects and the effects of male quality on parental investment in many systems, the consistency of these patterns has not been established and has hardly been investigated in *Anolis* systems. We found no effect of sire skin

coloration or body size on egg characteristics. Studies on barn swallows (2008), red-legged partridges (2012), great tits (Isaksson et al., 2006), and mallards (Giraudeau et al., 2011) have similarly found no effect of male coloration or quality on the carotenoid content of eggs, despite sometimes finding an eventual effect on offspring coloration. However, our result is unexpected since male body condition has been found to influence the sex ratio of offspring in this species (Cox et al., 2011), and thus it seemed likely that large or carotenoid-rich males may influence the maternal investment to eggs. However, as noted above, further work on the downstream effects on fitness (e.g. offspring survival) needs to be conducted to definitively determine if sire coloration and body size have a role in this system.

We found that *Anolis* dams may allocate carotenoids to eggs to improve maternal oxidative stress status based on the apparent dumping of excess carotenoids into unfertilized eggs, and the lack of an associated increase in the antioxidant activity of eggs laid by carotenoid-supplemented females. Additionally, we found no impact of paternal traits on the content or antioxidant capacity of eggs, but more work should be done before concluding that paternal effects do not have a role in the *Anolis* system. The hypothesis that the maternal allocation of carotenoids to eggs is at least partially to improve maternal oxidative stress status needs to be considered more widely and may explain results of other studies that found a limited effect or did not find an effect of carotenoid supplementation on offspring antioxidant capacity and fitness (e.g. Svensson et al., 2006; Hõrak et al., 2007; Grether et al., 2008).

Declaration of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Kevin McGraw, Craig Layne, Christine Urbanowicz, and Michelle Lawing for input and assistance with this project.

Funding

This work was supported by The Dartmouth College Cramer Fund. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under grant number DGE1313911.

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