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Laying-Sequence Variation in Yolk Carotenoids of Eastern Bluebirds

Jessica Barkhouse

COLUMBUS STATE UNIVERSITY

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A THESIS SUBMITTED TO THE

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IN PARTIAL FULFILLMENT OF THE

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JESSICA BARKHOUSE

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Approved by

Dr. Jennifer Newbrey, Committee Chair

Dr. Michael Newbrey, Committee Member

Dr. Cindy S. Ticknor, Committee Chair & Dean

Columbus State University

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Jessica Barkhouse

Abstract

Carotenoids are naturally occurring, fat-soluble pigments that play an important role in the embryonic development of songbirds. Female songbirds use different strategies to allocate these important maternal resources to their eggs. Little research has been done on whether North American songbirds exhibit laying sequence patterns in their allocation of carotenoids. We studied the laying-sequence variation in yolk carotenoids and egg metrics of nine full clutches of Eastern Bluebirds (*Sialia sialis*) in Columbus, Georgia. I predicted that Eastern Bluebirds would exhibit a brood reduction strategy, with the first-laid eggs containing a higher concentration of carotenoids than the last-laid eggs. I also predicted that the egg mass and size would remain consistent across the clutch due to an increase in secondary resources across the clutch. My results indicated that Eastern Bluebirds did not follow a brood reduction strategy but rather had unpredictable varying carotenoid concentrations across the clutch. I found five carotenoids in the egg yolks of Eastern Bluebirds: lutein, β-carotene, zeaxanthin, astaxanthin, and β-cryptoxanthin. As predicted, the egg mass, egg length, and egg width did not vary significantly across the clutch.

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Introduction

Female birds allocate many different types of resources to their egg yolks, including lipids, hormones, antibodies, and carotenoids. Carotenoids are fat-soluble yellow, orange, and red pigments that are synthesized by plants, algae, and photosynthetic bacteria (Yamaguchi 2010). There are over 600 naturally-occurring carotenoids that can be classified as either carotenes or xanthophylls based on their structure and corresponding color reflectance (Pfander 1992). Carotenes are hydrocarbons that are responsible for red and orange coloration in birds, whereas xanthophylls are oxidized hydrocarbon that are responsible for yellow coloration. Birds are unable to synthesize carotenoids, so they obtain carotenoids from their diet of seeds, fruits, and/or insects (Gowaty and Plissner 1998). These pigments play important roles in immunostimulation, antioxidation, free radical scavenging, sexual signaling, and color vision (Lim and Pike 2016, Krinsky et al. 2004). In addition to these functions, carotenoids also play an important role in embryonic development.

The rapid growth of avian embryos results in the production and accumulation of free radicals and reactive metabolites in the egg (Deeming and Pike 2013). These natural byproducts of metabolism can cause significant damage to DNA and can result in oxidative stress that inhibits the development of the immune system (Schantz et al. 1999). Carotenoids in the yolk function as free radical scavengers during lipid peroxidation in embryonic tissues as the yolk is being broken down and used as an energy source (Blount et al. 2000, Surai et al. 2001). Bird species with higher rates of embryonic development compensate for the high levels of oxidative stress by having a higher concentration of carotenoids and vitamin E in the yolk (Deeming and Pike 2013). Some yolk carotenoids are provitamins to important antioxidants, including β-carotene, a provitamin that converts into vitamin A under the right enzymatic and metabolic conditions (Surai 2002, Deeming and Pike 2013). Due to the important protective functions that carotenoids provide during embryonic development, carotenoid concentrations experienced in the egg can also have long-lasting post-hatching effects on birds.

In a study of the effect of yolk carotenoids on hatchlings in the Blue Tit (*Cyanistes caeruleus*), carotenoid supplemented females had nestlings with faster immune system development and brighter yellow feathers (Biard et al. 2005). The nestlings from supplemented females, however, did not differ significantly in body mass or plasma antioxidants compared to control nestlings. A study on Zebra Finches (*Taeniopygia guttata*) showed that mothers fed carotenoid-rich diets laid eggs with higher concentrations of α-tocopherol, demonstrating that the female birds allocated more carotenoids to their yolks when the pigments were available (Royle et al. 2003). Another study on Zebra Finches found that mothers fed a carotenoid-rich diet had a higher rate of hatchling survival (McGraw et al. 2005). Higher concentrations of carotenoids in egg yolks reduce the extent of free radicals damage during embryonic metabolism and growth, which increases the likelihood of hatchling survival.

Each bird species has a specific strategy for allocating resources, including carotenoids, to their eggs. Some species use a brood-reduction strategy and allocate most of the maternal resources to the first few eggs to give the first-hatched nestling a higher chance of survival. Other species, using a brood-survival strategy, allocate more secondary maternal resources to the last-laid eggs in order to give an equalized chance of survival across the clutch (Slagsvold et al. 1984). Two examples of bird species that follow a brood-survival strategy are the Red-Winged Blackbird (*Agelaius phoeniceus*) and the Blue Tit. The Red-Winged Blackbird allocates higher concentrations of secondary resources to the last few eggs in order to compensate for the survival disadvantage that occurs with asynchronous hatching, with the larger first-hatched chicks outcompeting the smaller chicks for food (Newbrey et al. 2014). This allocation strategy causes the last-laid egg in a Red-Winged blackbird clutch to be the largest in size, even though it has the lowest concentration of yolk carotenoids. The Blue Tit has also been shown to allocate more antioxidants to the first few eggs in the clutch (Royle et al. 2003). In the Blue Tit, egg size was not correlated with egg quality. The last-laid, largest egg had a lower concentration of lutein, canthaxanthin, β-cryptoxanthin, β-carotene, and vitamin E, but had higher concentrations of lipids and other secondary maternal resources. Conversely, in a brood-reduction strategy, the last egg is smaller and the mother does not invest as heavily in it. The Lesser Black-Backed Gull (*Larus fuscus*) allocates more carotenoids, lipids, and vitamin E to the first two eggs in a clutch (Royle et al. 2002). The last egg in the clutch is significantly smaller in size and has notably less maternal resources. In both the brood-reduction and brood-survival strategy, the decrease of carotenoids throughout the laying sequence indicates that there are limited maternal resources (Biard et al. 2005).

Egg size variation can also be an indication of what strategy the mother is using in allocating her resources. This is highly variable across species. The Ring-Billed Gull (*Larus delawarensis*), American Coot (*Fulica americana*), and Purple Heron (*Ardea purpurea*) all have decreasing egg metrics within a clutch (Arnold 1991, Jover et al. 1993, Meanthrel and Ryder 1987). However, in the Red-Winged blackbird, the egg size increases across the clutch (Newbrey et al. 2014). There are also birds, like Canada Geese (*Branta canadensis moffitti*), that lay the smallest eggs at the beginning and end of the clutch (Leblanc 1987). In some songbirds, including the Eastern Bluebird (*Sialia sialis*), the eggs become slightly heavier across the clutch (Gowaty and Plissner 1998).

For my senior research project, I studied the variation in yolk carotenoids across the laying-sequence in Eastern Bluebirds. Eastern Bluebirds are a particularly interesting study species because no prior research has been done to identify or quantify the yolk carotenoids of the species. Based on previous research, I hypothesized that Eastern Bluebirds would allocate carotenoids in a brood-reduction strategy. I predicted that the first few eggs would contain higher concentrations of carotenoids and that each consecutive egg would contain a lower concentration. Despite the decreasing carotenoid levels, I predicted that the females would allocate secondary resources to keep the egg size and egg mass fairly constant across a clutch. I collected full clutches of eggs from female Eastern Bluebirds nesting in nest boxes on the main campus of Columbus State University in Columbus, GA.

Methods

Study Species

The Eastern Bluebird (*Sialia sialis*) is a sexually dimorphic songbird that is widely distributed across the eastern half of the United States. Males and females are easily distinguishable through their coloration. Males have deep blue upper parts with a red orange breast and white lower belly. Females are much more subdued in color, with dull blue grey upper parts and light orange breasts (Gowaty and Plissner 1998). As partial migrants, only a portion of the Eastern Bluebird population migrates to a warmer climate during winter months (Gowaty and Plissner 1998). They feed primarily on insects such as grasshoppers, caterpillars, beetles, and crickets mainly by drop foraging. During the breeding season, they perch anywhere from 0.5 m to 15 m above the ground to spot ground arthropods, sometimes up to 40 m away.

During the winter, bluebirds mostly feed on small fruits like the berries of flowering holly, dogwood, or mulberry by perching on stalks of fruiting bushes or trees (Taylor 2017).

The Eastern Bluebird nests in naturally-occurring or man-made cavities, preferably in open habitats with minimal ground cover (Gowaty and Plissner 1998). They are highly territorial birds with increased defense of their home ranges (on average ~2 ha) during the breeding season. Although they are typically socially monogamous, extra-pair copulations do occur. This is advantageous for bluebirds because it increases the genetic diversity within clutches. While males have been observed guarding and feeding their pair-bonded females to prevent them from engaging in extra-pair copulations, they are not always successful. In fact, one study found that 24% of broods contained one or more extra-pair offspring (Meek et al. 1994). Males can also aid in feeding the hatchlings, especially at later times in the day (Pinkowski 1978).

Females construct cup nests made primarily of grass and pine needles (Gowaty and Plissner 1998). Clutch size ranges from 3-7 eggs with an incubation that lasts, on average, 2 weeks. Because incubation begins after the last egg is laid, bluebirds have synchronous hatching. This results in higher rates of survival for all the chicks in a clutch. The eggs are baby blue in color and average 21 mm in length. While the size of the eggs remains relatively constant, the mass of the eggs increases across the laying sequence. The first few eggs in a clutch average 3.01 g while the last few weigh 3.1 g. Females can produce three or more successful broods per season, especially in southern populations (Gowaty and Plissner 1998). Hatchlings are fed a diet of lepidoptera larvae, orthopterans, spiders, and fleshy fruits (Pinkowski 1978).

Study Site

This study was conducted on the main campus of Columbus State University (CSU). Established in 1958, CSU's Main Campus has 53.4 ha with sports facilities, parking lots, dorms, fields, and classroom buildings. There are a total of 51 nest boxes distributed around the campus, some in quieter, more secluded areas and others closer to roads and foot traffic.

Field Methods

Starting in May 2019, I checked nest boxes weekly. I collected full clutches of eggs from the second or third clutch of each female to reduce the chances of nest abandonment and to encourage females to renest in the same boxes. I checked active nests without eggs every other day. Once the first egg was laid, I checked the nest every day. The eggs were marked on the day they were laid using a non-toxic marker and were collected the following day. Leaving at least one egg in the nest at all times helped prevent the mother from abandoning her nest prior to clutch completion.

Laboratory Methods

When brought into the lab, each egg's total mass (to the nearest 0.01 g), length, and width were measured. Eggs were then wrapped in Parafilm and placed in the freezer at 20°C. While the eggs were still partially frozen, the albumen was separated from the yolk. The mass of the yolk and shell was determined and a subsample of the yolk (~120 mg) was isolated for carotenoid extraction. Each sample contained the subsample of the yolk, two 1-mm glass beads, and 10 μL of capsanthin, an internal standard. Then, 500 mL of 5% NaCl was added to each sample and they were vortexed for 2 minutes. A 900 mL solution of 10% isopropyl alcohol in hexane was added, the sample was vortexed for 2 minutes and centrifuged for 5 minutes at 8,000 rpm, and the organic fraction was drawn out using a pipet. These steps were repeated, and both organic fractions were combined and filtered through 0.2 µm syringe filters. The carotenoid extract was injected into an Agilent 1220 high-performance liquid chromatography (HPLC) system fitted with a normal phase silica column with 100 Å pore sizes (250×4.6 mm; Phenomenex, Torrance, California).

Statistical Analysis

The relationships between egg mass, length, width, individual carotenoid concentration, combined carotenoid concentrations, and laying sequence were analyzed using linear mixed effects model in R software (R Core Team) and lme4 (Bates et al. 2013). Laying order was included as a fixed effect and female identity was included as a random effect. We used a likelihood ratio test, where the null model did not include laying order, to determine our P value.

Results

In total, I analyzed the carotenoids in nine full clutches of Eastern Bluebird eggs, totaling 36 eggs. I identified five carotenoids in the egg yolks of Eastern Bluebirds; lutein was the most abundant, followed by β-carotene, zeaxanthin, astaxanthin, and lastly β-cryptoxanthin (Table 1). According to my results, there was not a significant difference in yolk carotenoids across the laying sequence when measured individually or when all carotenoids were combined (Table 2). Additionally, there was not a significant difference in egg mass, egg length, or egg width across the clutch (Table 2).

Discussion

I identified five carotenoids, lutein, β-carotene, zeaxanthin, astaxanthin, and β-cryptoxanthin, in the egg yolks of Eastern Bluebirds. The most abundant carotenoid was lutein, with concentrations averaging ten times higher than the concentrations of the second most abundant carotenoid, β-carotene. Lutein is commonly one of the highest concentrated carotenoids in the egg yolks of songbirds from the Old World, including the Pied Flycatcher (*Ficedula hypoleuca*) and the Blue Tit (Eeva et al. 2011, Biard et al. 2005). In the few New World species that have been studied, the most abundant carotenoid is not as easily predictable. In the Red-Winged Blackbird, the most abundant carotenoid was α-doradexanthin, a converted red carotenoid (Newbrey et al. 2014). The yolks of Yellow-Headed Blackbirds (*Xanthocephalus xanthocephalus*), another New World species, contained β-carotene as the most abundant carotenoid (Newbrey et al. 2008). Still other New World species, like the House Finch (*Haemorhous mexicanus*), contained lutein as their most abundant carotenoid (McGraw et al. 2008).

Astaxanthin is a converted, red keto-carotenoid that is often present in the yolks of birds with red plumage, like the Red-Winged Blackbird (Newbrey et al. 2014). It has not been identified in yolks of other New or Old-World species that do not contain red pigmentation in their feather coloration. Therefore, the presence of astaxanthin in the egg yolks of Eastern Bluebirds was surprising since they do not display this red carotenoid in their plumage. Astaxanthin may be present in the yolks as a result of the diet of Eastern Bluebirds, but it may also play an undiscovered role in their development.

My results showed no significant variation in yolk carotenoids across the eggs of a clutch. This suggests that Eastern Bluebirds may not exhibit a brood-reduction strategy like I had hypothesized. This was surprising because other passerine species, including the Red-Winged Blackbird, Yellow-Headed Blackbird, and Blue Tit, exhibit brood-reduction strategies in their laying sequence (Newbrey et al. 2014, Newbrey et al. 2008, Royle et al. 2003). Interestingly, four out of the nine clutches that we collected did show decreasing total carotenoids across the clutch. This suggests that a larger sample size may provide a more accurate pictures of how Eastern Bluebirds allocate their resources to their eggs. Our results also showed no significant variation in egg metrics across the clutch. This was unexpected because previous studies had found a decrease in egg mass across the clutch (Gowaty and Plissner 1998).

In conclusion, my study on the variation in carotenoids across the laying sequence of Eastern Bluebirds indicated that there was not a consistent strategy used to allocate these maternal resources. We found five carotenoids in the egg yolks of bluebirds: lutein, β-carotene, zeaxanthin, astaxanthin, and β-cryptoxanthin. The presence of astaxanthin was surprising because of the lack of red feather coloration in Eastern Bluebirds. Further research could focus on the role astaxanthin has in bluebird development and whether any evolutionary advantages exist in allocating energy to produce it. Further research could also focus on collecting a larger sample size of full clutches to better understand how Eastern Bluebirds allocate maternal resources to their eggs.

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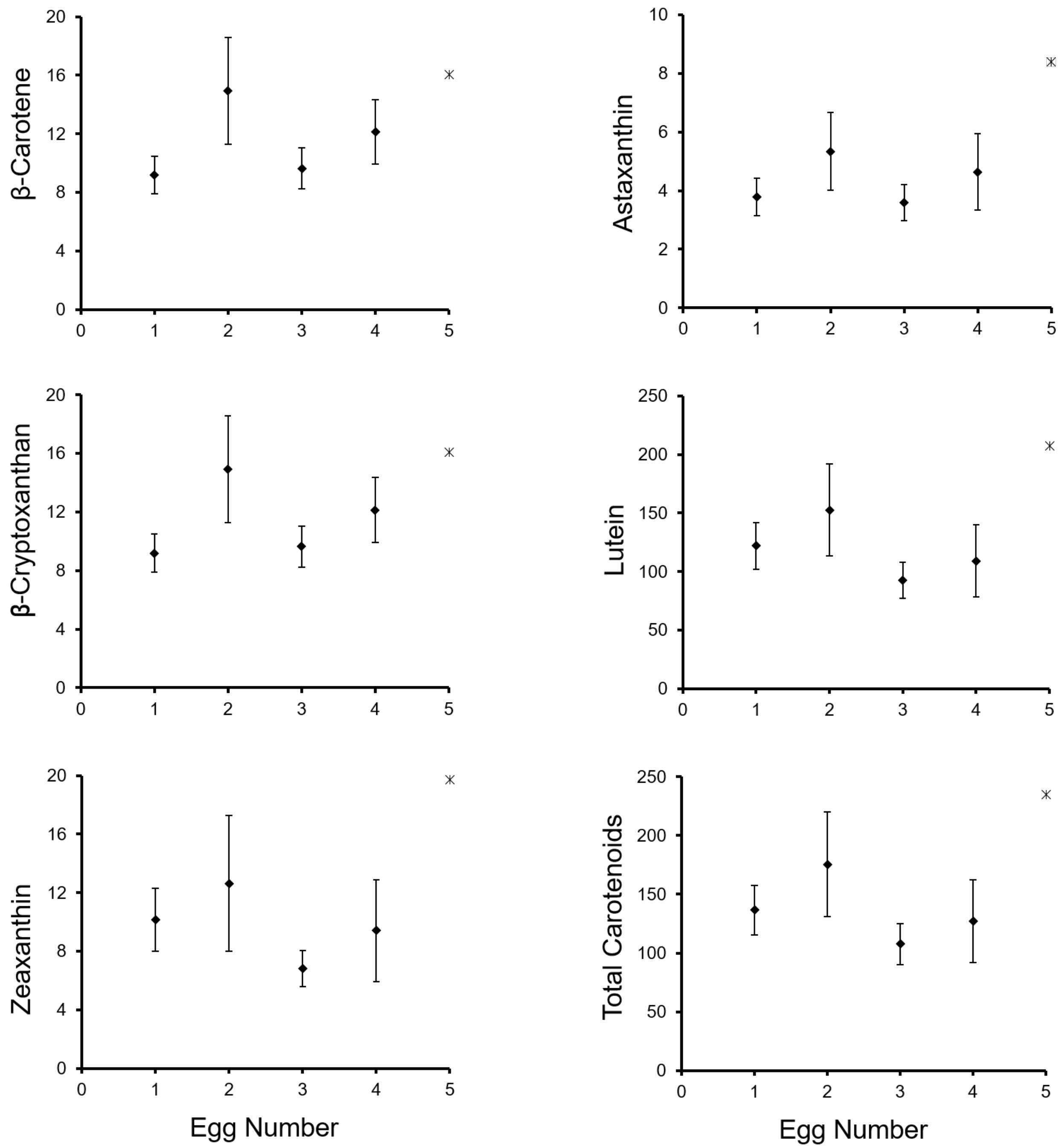

Figure 1. Concentrations (μg/g yolk) of β-carotene, β-cryptoxanthan, zeaxanthin, astaxanthin, lutein, and total carotenoids in first- to fourth-laid eastern bluebird eggs. Values are mean ± 1 SE. * = values for one fifth-laid egg.

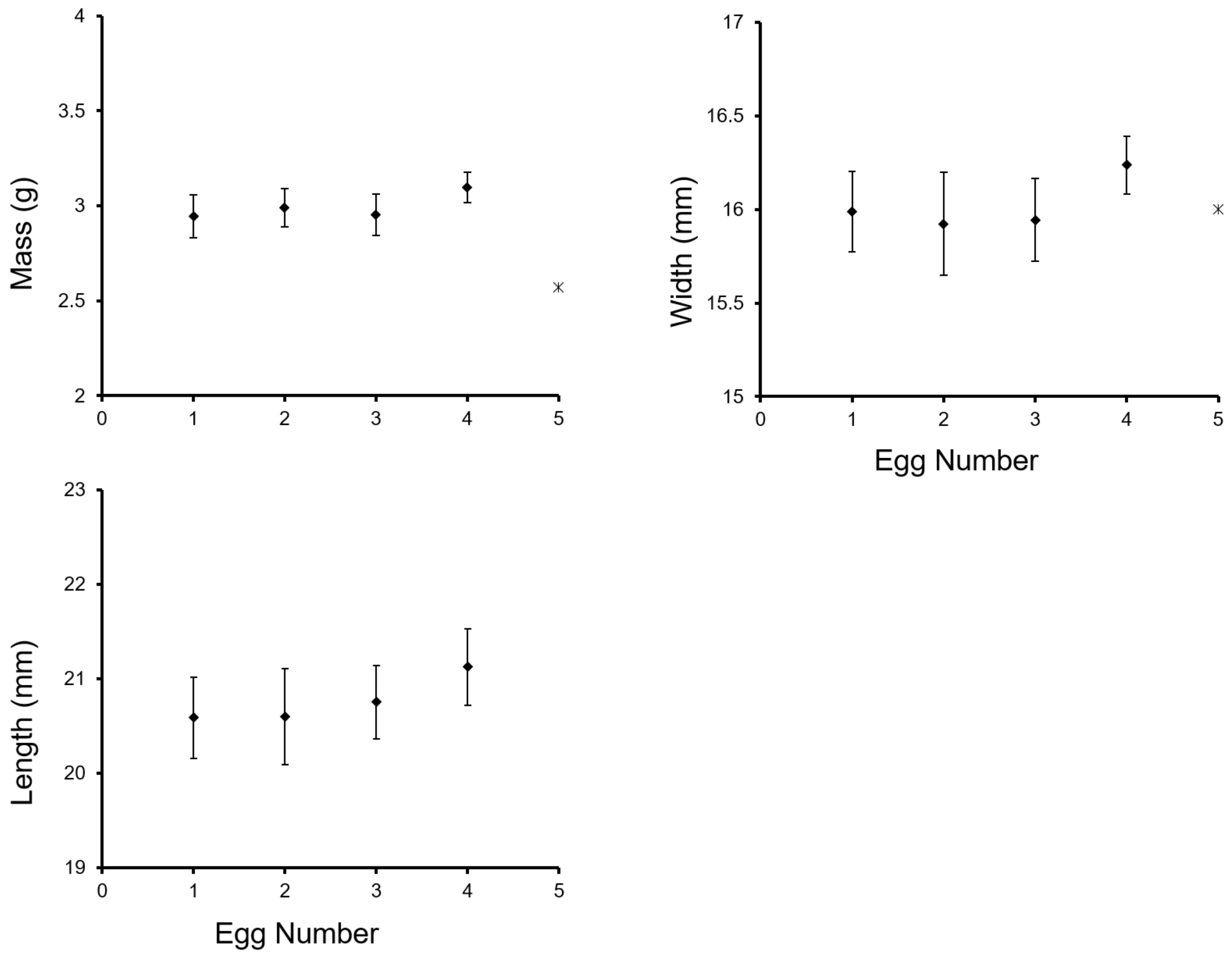

Figure 2. Variation in egg mass, width, and length of Eastern Bluebird eggs in first- to fourth-laid eggs. Values are mean ± 1 SE. * = values for one fifth-laid egg.

	Egg 1 (n=9)	Egg 2 (n=9)	Egg 3 (n=9)	Egg 4 (n=8)
Egg mass (g)	2.9 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	3.1 ± 0.1
Egg width (mm)	15.9 ± 0.2	15.9 ± 0.3	15.9 ± 0.2	16.2 ± 0.2
Egg length (mm)	20.6 ± 0.4	20.6 ± 0.5	20.8 ± 0.4	21.1 ± 0.4
β -carotene (μg/g yolk)	9.2 ± 1.2	11.9 ± 2.5	9.7 ± 1.4	12.1 ± 2.2
β -cryptoxanthan (μg/g yolk)	1.6 ± 0.2	2.2 ± 0.8	1.8 ± 0.7	2.4 ± 0.9
Zeaxanthin (μg/g yolk)	10.2 ± 2.2	8.5 ± 1.3	6.8 ± 1.2	9.4 ± 3.5
Astaxanthin (μg/g yolk)	3.8 ± 0.6	4.0 ± 0.7	3.6 ± 0.6	4.6 ± 1.3
Lutein (μg/g yolk)	121.9 ± 20.0	114.4 ± 16.5	92.6 ± 15.3	109.1 ± 30.7
Total Carotenoids (μg/g yolk)	136.4 ± 21.0	133.5 ± 44.4	107.7 ± 17.4	127.0 ± 35.1

Table 1. Variation in egg metrics and yolk carotenoid concentrations across the laying sequence in eggs of Eastern Bluebirds. Values are mean ± 1 SE.

	p	DF	X^2
Egg mass	0.367	1	0.815
Egg width	0.883	1	0.022
Egg length	0.141	1	2.18
β -carotene	0.521	1	0.413
β -cryptoxanthan	0.355	1	0.551
Zeaxanthin	0.425	1	0.636
Astaxanthin	0.647	1	0.21
Lutein	0.474	1	0.513
Total Carotenoids	0.563	1	0.336

Table 2. Results of linear mixed effects models and likelihood ratio tests analyzing relationships between the fixed effect (laying order), random effect (female identity), yolk carotenoid concentration, and egg metrics of Eastern Bluebird eggs. Values are mean ± 1 SE.