



## LAYING-SEQUENCE VARIATION IN YOLK CAROTENOID CONCENTRATIONS IN EGGS OF YELLOW-HEADED BLACKBIRDS (*XANTHOCEPHALUS XANTHOCEPHALUS*)

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**ABSTRACT.**—Asynchronous hatching creates a size hierarchy among siblings and a survival disadvantage for last-hatched nestlings. Female birds can influence this disadvantage by differentially investing maternal resources, such as carotenoids, across the laying sequence. We studied intraclutch variation in carotenoid concentrations in Yellow-headed Blackbirds (*Xanthocephalus xanthocephalus*) and predicted that yolk concentrations would decrease across the laying sequence, because nestling mortality is inversely related to hatching order in this species. We quantified intraclutch variation in the concentrations of total and individual identifiable carotenoids ( $\beta$ -carotene,  $\beta$ -cryptoxanthin, lutein, and zeaxanthin) in Yellow-headed Blackbird eggs collected from five breeding colonies, and correlated these concentrations with egg mass, yolk mass, and yolk water content. Carotenoid concentrations were not related significantly to any of the egg metrics measured. The concentration of total identifiable carotenoids increased across the laying sequence in Yellow-headed Blackbird eggs, which is the opposite of what has been found in most other passerines. Concentrations of the two most abundant carotenoids,  $\beta$ -carotene and  $\beta$ -cryptoxanthin, and zeaxanthin, all increased across the laying sequence, whereas the concentration of less-abundant lutein decreased. If yolk carotenoid concentrations contribute to increased survival of older over younger Yellow-headed Blackbird nestlings, concentrations of specific carotenoids, such as lutein, may be important. The differential change in concentrations of the various carotenoids across Yellow-headed Blackbird clutches may be attributable to the availability of these compounds in the diet combined with differences in absorption and antioxidant function. Received 14 September 2006, accepted 29 March 2007.

Key words:  $\beta$ -carotene,  $\beta$ -cryptoxanthin, laying sequence, lutein, *Xanthocephalus xanthocephalus*, Yellow-headed Blackbird, zeaxanthin.

### Variation des concentrations en caroténoïde vitellin dans les œufs de *Xanthocephalus xanthocephalus* selon la séquence de ponte

**RESUME.**—L'asynchronisme des éclosions crée une hiérarchie dans la taille des membres d'une même couvée et un désavantage pour la survie des poussins éclos les derniers. Les oiseaux femelles peuvent influencer sur ce désavantage en investissant leurs ressources maternelles, telles que les caroténoïdes, de façon différentielle dans la séquence de ponte. Nous avons étudié la variation intra-couvée des concentrations en caroténoïdes chez *Xanthocephalus xanthocephalus*. Nous avons émis l'hypothèse que les concentrations en caroténoïdes vitellins diminuent à dans la séquence de ponte car la mortalité des poussins est inversement reliée à l'ordre d'éclosion chez cette espèce. Nous avons quantifié la variation intra-couvée des concentrations en caroténoïdes identifiables totaux et individuels ( $\beta$ -carotène,  $\beta$ -cryptoxanthine, lutéine et zéaxanthine) dans des œufs récoltés dans cinq colonies de cette espèce. Nous avons effectué des corrélations entre ces concentrations et la masse de l'œuf, la masse du vitellus et le contenu en eau du vitellus. Il n'y avait pas de relation significative entre les concentrations en caroténoïdes et les mesures réalisées sur les œufs. La concentration en caroténoïdes identifiables totaux s'est accrue avec l'ordre de ponte des œufs de *X. xanthocephalus*, contrairement à ce qui a été trouvé chez la plupart des passereaux. Les concentrations de la zéaxanthine et des deux caroténoïdes les plus abondants, soit le  $\beta$ -carotène et la  $\beta$ -cryptoxanthine, ont toutes augmenté dans la séquence de ponte alors que la concentration du moins abondant, la lutéine, a diminué. Si les concentrations en caroténoïdes vitellins contribuent à augmenter la survie des poussins premiers éclos en comparaison des derniers éclos, les concentrations en caroténoïdes spécifiques, telles que la lutéine, peuvent être importantes. Le changement différentiel dans les concentrations en caroténoïdes dans les couvées de *X. xanthocephalus* peuvent être attribuables à la disponibilité de ces composés dans le régime alimentaire et aux différences d'absorption et de la fonction anti-oxydante.

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HATCHING ASYNCHRONY, a common hatching strategy among altricial birds, creates a size hierarchy among siblings and a survival disadvantage for last-hatched nestlings (Stenning 1996). Female birds can influence this survival disadvantage by differentially allocating maternal resources, such as lipids (e.g., Royle et al. 1999), androgens (e.g., Groothuis and Schwabl 2002), antibodies (e.g., Blount et al. 2002), and carotenoids (e.g., Hōrak et al. 2002), across the laying sequence. Carotenoids are biologically active, lipid-soluble, yellow and red pigments synthesized by plants and photosynthetic microorganisms, which birds obtain from their diet (Blount et al. 2000). The maternal allocation of carotenoids to eggs affects both embryonic development and posthatching health and survival. In the egg, yolk carotenoids protect embryonic tissues against metabolic free radicals, which can damage lipids, proteins, and DNA (Edge et al. 1997, Møller et al. 2000). Egg-yolk carotenoid concentrations also affect offspring performance after hatching. Lesser Black-backed Gull (*Larus fuscus*) chicks that hatched from eggs with low carotenoid concentrations suffered higher mortality in the first few days after hatching than chicks from eggs with high concentrations (Royle et al. 2001). Biard et al. (2005) also found that nestling Blue Tits (*Cyanistes caeruleus*) from eggs with higher concentrations of carotenoids were larger and had faster-developing immune systems than nestlings from eggs with lower carotenoid concentrations.

Intraclutch variation in yolk carotenoid concentrations has been observed in several bird species. Captive Zebra Finches (*Taeniopygia guttata*) allocate more carotenoids to their later-laid eggs, which is correlated with an increased probability of survival for later-hatched chicks (Williamson et al. 2006). By contrast, Lesser Black-backed Gulls (Royle et al. 2001, Blount et al. 2002), Barn Swallows (*Hirundo rustica*; Saino et al. 2002), Great Tits (*Parus major*; Hōrak 2002), Red-legged Partridges (*Alectoris rufa*; Bortolotti et al. 2003), and eight introduced passerines in New Zealand (Cassey et al. 2005) allocate higher concentrations of carotenoids to their earlier-laid eggs than to later-laid eggs. In such cases, it is believed that carotenoids are limited and that females deplete their carotenoid stores as they lay more eggs (Saino et al. 2002). Several studies have provided evidence to support the “carotenoid-limitation” hypothesis. Blount et al. (2004) found that dietary carotenoid availability limited the relaying capacity of female Lesser Black-backed Gulls after their clutches were removed. Also, female Lesser Black-backed Gulls (Blount et al. 2002), Blue Tits (Biard et al. 2005), and Hihi (*Notiomystis cincta*; Ewen et al. 2006), when fed carotenoid-enriched diets, lay eggs with higher concentrations of carotenoids than control birds.

Laying-sequence variation in carotenoid concentrations has been described for only a handful of passerine species, and most studies have focused on either one representative carotenoid or total carotenoids. Here, we quantified laying-sequence variation in the concentrations of total and individual identifiable carotenoids ( $\beta$ -carotene,  $\beta$ -cryptoxanthin, lutein, and zeaxanthin) in full clutches of eggs of Yellow-headed Blackbirds (*Xanthocephalus xanthocephalus*). Nestling mortality is inversely related to hatching order in this species (Willson 1966), with last-hatched nestlings surviving only when earlier-hatched nestlings die (Barber and Evans 1995), which suggests that Yellow-headed Blackbirds practice adaptive brood reduction (Forbes et al. 2002). On the basis of the life history of Yellow-headed Blackbirds and the results

of previous studies of laying-sequence variation in carotenoid concentrations, we predicted that earlier-laid eggs would have higher concentrations of carotenoids than later-laid eggs.

Carotenoid concentrations are reported to be either positively or negatively correlated with egg size, depending on the species studied. Carotenoid concentration is positively related to egg size in Common Moorhens (*Gallinula chloropus*; Fenoglio et al. 2003) and Lesser Black-backed Gulls (Royle et al. 2001), but negatively related to egg size in eight nonnative bird species in New Zealand (Cassey et al. 2005). Therefore, we measured egg mass, yolk mass, and yolk water content of Yellow-headed Blackbird eggs to determine their relationship to carotenoid content. We predicted that there would be a decrease in egg and yolk mass across the laying sequence, and a positive relationship between egg mass and carotenoid concentration, because larger eggs represent a larger investment of resources by the laying female.

## METHODS

*Study species and field methods.*—Eggs were collected from five free-living breeding colonies of Yellow-headed Blackbirds in Stutsman County, North Dakota, 19–29 May 2004 and 2005. Yellow-headed Blackbirds breed in prairie wetlands and have a polygynous mating system with typically one to six females in a male’s territory. They are determinant layers, with a normal clutch size of four eggs (Twedt and Crawford 1995). Most females begin incubating after their second egg is laid, resulting in synchronous hatching of the first and second egg, but asynchronous hatching of the third and fourth egg (Twedt and Crawford 1995), which creates a survival disadvantage for the last-hatched chick (Barber and Evans 1995). Yellow-headed Blackbirds produce only one brood per season, but some females renest if their first nest attempt fails early in the breeding season (Twedt and Crawford 1995). Juvenile birds do not obtain yellow plumage until they have completed a prebasic molt at ~80 days of age (Twedt and Crawford 1995).

Active nests were located within each breeding colony, and if an egg was present, it was marked with a small dot of nontoxic black ink on the blunt end to indicate laying sequence. Full clutches were collected from 13 nests in 2004 and 11 nests in 2005 for a total of 87 eggs: 24 first-laid eggs, 24 second-laid eggs, 22 third-laid eggs, and 17 fourth-laid eggs. Second-, third-, and fourth-laid eggs were collected on the day they were laid to prevent variation in carotenoid concentration because of embryonic metabolism. The first-laid egg was collected at the same time as the second-laid egg in light of the delayed onset of incubation. Collected eggs were replaced with marked eggs from nests not studied so that at least one egg was present in each study nest at all times. Collected eggs were kept on ice  $\leq 6$  h before each egg was weighed to the nearest 0.1 g, wrapped in Parafilm, and frozen at  $-20^{\circ}\text{C}$  until analysis.

*Laboratory methods.*—Yolks were separated from the shells and albumen, weighed to the nearest 0.01 g, and homogenized to eliminate variation in carotenoid concentration across layers; small subsamples of known mass (100–150 mg) were removed for carotenoid extraction. Yellow-headed Blackbird yolks vary in water content (43–65%), so subsamples were dried at  $35^{\circ}\text{C}$  for 3 h in a CentriVap centrifugal concentrator (Labconco, Kansas City, Missouri). The dry yolk samples were broken into fine particles,

and two 1-mm glass beads and 4.22  $\mu\text{g}$  of an internal standard (astaxanthin; Sigma-Aldrich, St Louis, Missouri) were added to each sample. Astaxanthin was used as an internal standard because it was not found in Yellow-headed Blackbird yolks in preliminary analyses, it absorbs strongly at 450 nm, and it eluted at a retention time distinct from that of all other 450-nm-absorbing components of yolk (Fig. 1). To determine whether drying influenced the carotenoid content of yolk samples, we extracted and quantified carotenoids from dried and wet subsamples of five yolks and compared carotenoid concentrations.

The method for carotenoid extraction was modified from a previously published procedure (Surai et al. 2001). In short, 500  $\mu\text{L}$  5% NaCl(aq) solution was added to a yolk sample, vortexed for 2 min, 1 mL 10% (v/v) isopropyl alcohol in hexane was added, and the sample was vortexed for an additional 2 min. The sample was centrifuged for 5 min at  $6,000 \times g$  before the organic layer containing the carotenoids was collected. The procedure was repeated, and the two organic fractions were combined and filtered through 2  $\text{\AA}$  syringe filters (National Scientific, Rockwood, Tennessee). To determine the amount of variation associated with the extraction method, three yolks from nonstudy eggs were divided into five subsamples and extracted as above, and carotenoids were quantified (see below).

Twenty microliters of each sample was injected into a Shimadzu SCL-6A high-performance liquid chromatography (HPLC) system fitted with a normal phase silica column (100  $\text{\AA}$ ;  $250 \times 4.6$  mm; Phenomenex, Torrance, California) with a mobile phase of 4.5% (v/v) isopropyl alcohol in hexane at a constant flow rate of  $1 \text{ mL min}^{-1}$ . These conditions allowed good separation of lutein and zeaxanthin (Panfili et al. 2004). Peaks were detected by UV absorption at 450 nm. Beta-carotene,  $\beta$ -cryptoxanthin, lutein, and zeaxanthin peaks were identified by comparing their retention times with those obtained from authentic standards ( $\beta$ -carotene: Calbiochem, San Diego, California;  $\beta$ -cryptoxanthin: Indofine, Hillsborough, New Jersey; lutein: Sigma-Aldrich; zeaxanthin: Indofine). To determine the amount of variation associated with our HPLC quantification methods, we quantified carotenoids in two separate 20- $\mu\text{L}$  subsamples of three yolk extractions.

*Statistical analysis.*—General linear models (GLMs) were used to determine whether there were significant differences in the concentrations of total and individual carotenoids across the laying sequence. Female identity was used as a random effect in all models, because eggs laid by the same female were not independent of each other. Year was used as a covariate, because eggs were collected during two different breeding seasons. All GLMs were followed by Bonferroni multiple-comparison tests to determine pairwise differences. General linear models were also used to determine whether egg mass, yolk mass, and yolk water content varied from first- to fourth-laid eggs. Before running all GLMs, carotenoid concentrations and egg metrics were converted into  $z$ -scores using the following equation from Fowler et al. (1998):

$$Z = \frac{(\text{observation} - \text{clutch mean})}{\text{clutch standard deviation}}$$

Using  $z$ -scores allowed comparisons between all clutches by standardizing the carotenoid concentrations and metrics of each egg by its deviation from the mean of its clutch (i.e., clutch mean = 0, clutch SD = 1).

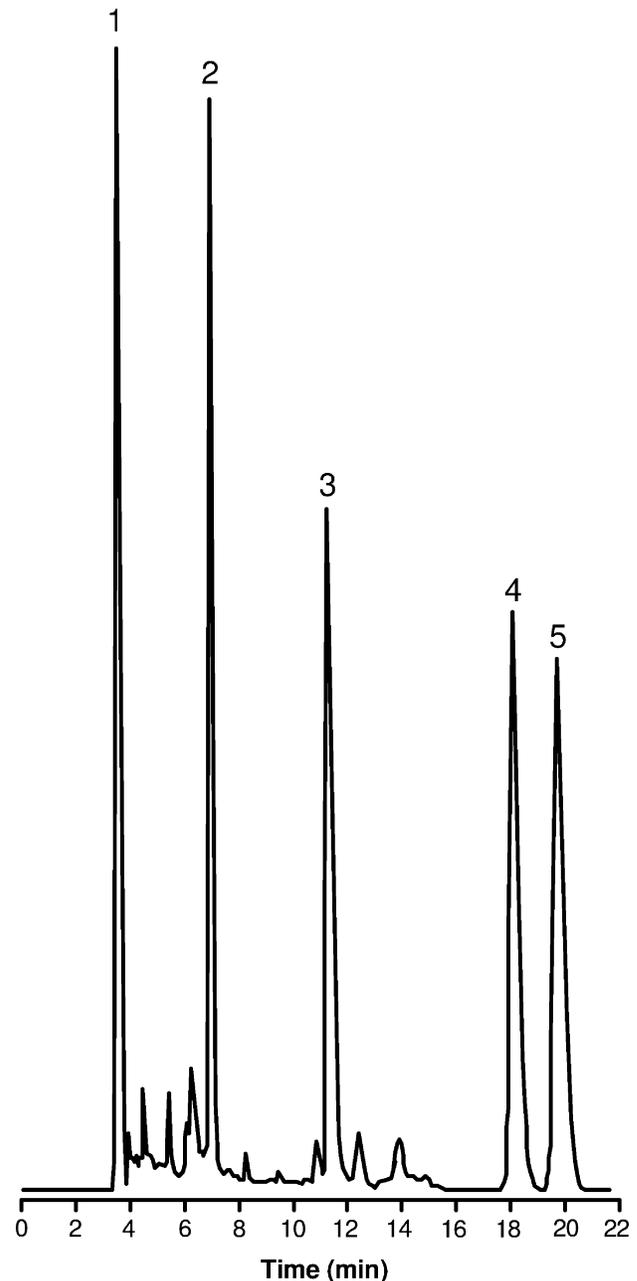


FIG. 1. HPLC chromatogram of carotenoids found in a typical Yellow-headed Blackbird yolk. Separation was done using normal phase with detection at 450 nm. Peak 1 =  $\beta$ -carotene, peak 2 =  $\beta$ -cryptoxanthin, peak 3 = astaxanthin (internal standard), peak 4 = lutein, and peak 5 = zeaxanthin.

To determine whether there were positive or negative correlations between the concentrations of the different carotenoids and egg metrics, data were analyzed using linear regression. Paired  $t$ -tests were used to compare carotenoid concentrations in wet and dry subsamples. All analyses were performed using the statistical program SYSTAT, version 11 (Systat Software, Richmond, California), with  $\alpha = 0.05$ .

TABLE 1. Variation in concentrations of yolk carotenoids and egg metrics across the laying sequence in Yellow-headed Blackbird clutches. Values are means  $\pm$  SE.

	Egg 1	Egg 2	Egg 3	Egg 4
Total carotenoids ( $\mu\text{g g}^{-1}$ yolk)	143.9 $\pm$ 6.3	151.7 $\pm$ 5.7	166.1 $\pm$ 6.6	174.5 $\pm$ 6.9
$\beta$ -carotene ( $\mu\text{g g}^{-1}$ yolk)	44.2 $\pm$ 2.7	51.7 $\pm$ 2.7	60.3 $\pm$ 3.1	65.7 $\pm$ 3.1
$\beta$ -cryptoxanthin ( $\mu\text{g g}^{-1}$ yolk)	47.7 $\pm$ 3.6	53.9 $\pm$ 3.1	64.1 $\pm$ 4.0	64.6 $\pm$ 4.0
Lutein ( $\mu\text{g g}^{-1}$ yolk)	24.9 $\pm$ 2.0	17.6 $\pm$ 1.4	12.9 $\pm$ 1.0	11.2 $\pm$ 1.2
Zeaxanthin ( $\mu\text{g g}^{-1}$ yolk)	28.7 $\pm$ 0.9	28.4 $\pm$ 0.9	31.2 $\pm$ 1.0	33.0 $\pm$ 1.3
Egg mass (g)	4.4 $\pm$ 0.1	4.5 $\pm$ 0.1	4.4 $\pm$ 0.1	4.4 $\pm$ 0.1
Yolk mass (g)	0.72 $\pm$ 0.02	0.74 $\pm$ 0.02	0.72 $\pm$ 0.02	0.73 $\pm$ 0.02
Yolk water (mg)	62.8 $\pm$ 1.3	60.9 $\pm$ 1.5	58.3 $\pm$ 1.3	60.7 $\pm$ 1.3

## RESULTS

**Laboratory methods.**—There were no significant differences in the concentrations of any of the carotenoids quantified between wet and dry samples (paired *t*-tests,  $P \geq 0.48$ ). The amount of variation between carotenoid extractions was low for the three eggs divided into five subsamples:  $\beta$ -carotene (CV = 8.8%),  $\beta$ -cryptoxanthin (CV = 8.5%), lutein (CV = 8.7%), and zeaxanthin (CV = 9.3%). The amount of variation explained by the carotenoid quantification method was low for all three yolk samples that were quantified twice ( $\beta$ -carotene CV = 1.0%,  $\beta$ -cryptoxanthin CV = 1.2%, lutein CV = 2.0%, zeaxanthin CV = 3.1%).

**Carotenoid concentrations.**—Linear regression showed that the concentrations of the four yolk carotenoids were not correlated ( $P > 0.05$ ) with egg mass, yolk mass, or yolk water content. Neither the covariate year nor the random factor, female identity, had significant coefficients in any of the GLMs ( $P > 0.05$ ). Total carotenoid concentrations varied across the laying sequence, with the first and second eggs having significantly lower concentrations of combined carotenoids than the third and fourth eggs ( $F = 11.75$ ,  $df = 83$ ,  $P < 0.001$ ; Table 1 and Fig. 2). The concentrations of  $\beta$ -carotene ( $F = 54.37$ ,  $df = 83$ ,  $P < 0.001$ ),  $\beta$ -cryptoxanthin ( $F = 22.15$ ,  $df = 85$ ,  $P < 0.001$ ), and zeaxanthin ( $F = 13.75$ ,  $df = 85$ ,  $P < 0.001$ ) were also lowest in first-laid eggs and highest in later-laid eggs. By contrast, the concentration of lutein was highest in first-laid eggs and lowest in later-laid eggs ( $F = 90.99$ ,  $df = 85$ ,  $P < 0.001$ ). None of the egg metrics measured varied significantly across the laying sequence ( $P > 0.05$ ; Table 1 and Fig. 3).

## DISCUSSION

Yellow-headed Blackbird eggs contain many of the same yellow carotenoid pigments found in the yolks of other passerines, but they are in different proportions. In all other passerine species, lutein and zeaxanthin are the most concentrated yolk carotenoids, and  $\beta$ -carotene and  $\beta$ -cryptoxanthin are found in lower concentrations (Biard et al. 2005, Cassey et al. 2005, Ewen et al. 2006, Hargitai et al. 2006). By contrast, Yellow-headed Blackbirds produce yolks with higher concentrations of  $\beta$ -cryptoxanthin and  $\beta$ -carotene and lower concentrations of zeaxanthin and lutein (Table 1).

The concentration of total identifiable carotenoids increases across the laying sequence in Yellow-headed Blackbird clutches, which is the opposite of what has been found in most other

passerines. Third and fourth Yellow-headed Blackbird eggs have significantly more combined carotenoids than the first and second eggs, a trend previously reported only in captive Zebra Finches (Williamson et al. 2006). All other passerines, including Barn Swallows (Saino et al. 2002), Great Tits (Hörak et al. 2002), and eight introduced passerines in New Zealand (Cassey et al. 2005) have an intraclutch decrease in carotenoid concentrations, which has been attributed to a limitation of carotenoids in their diets.

Three of the four individual carotenoids— $\beta$ -carotene,  $\beta$ -cryptoxanthin, and zeaxanthin—also increase in concentration across the laying sequence. These three carotenoids are part of the same carotenoid biosynthetic pathway in plants (lycopene  $\rightarrow$   $\beta$ -carotene  $\rightarrow$   $\beta$ -cryptoxanthin  $\rightarrow$  zeaxanthin; Pogson et al. 1996), which may help to explain why they have similar patterns of allocation; they are most likely found in the same dietary items. Lutein, which is part of a different branch of the pathway (lycopene  $\rightarrow$   $\alpha$ -carotene  $\rightarrow$  zeinoxanthin  $\rightarrow$  lutein; Pogson et al. 1996), is the only carotenoid that decreases across the laying sequence, and it is found in lower concentrations than the other three, which suggests that it is more limited in the diet. Lutein has long been established as an important antioxidant in bird eggs (Goodwin 1984) and is the major carotenoid found in the eggs of the Barn Swallow (Saino et al. 2002), Collared Flycatcher (*Ficedula albicollis*; Hargitai et al. 2006), Blue Tit (Biard et al. 2005), Hibi (Ewen et al. 2006), and eight bird species in New Zealand (Cassey et al. 2005). The concentration of lutein in Barn Swallow clutches also declines with laying order, which Saino et al. (2002) attributed to a progressive depletion of maternal lutein and a limited availability of lutein in the diet.

The carotenoid-specific concentration patterns in Yellow-headed Blackbird clutches also suggest that there may be limitations in the allocation of carotenoids to the egg yolk because of differences in absorption, function, or both. Carotenoids interact with one another during absorption across the gut (van den Berg 1999), with specific carotenoids accumulating in the blood and tissues at ratios different from those found in the diet (Goodwin 1984, White et al. 1993, Kostic et al. 1995). Differences in polarity between the carotenoid molecules appear to play a role in their absorption in the intestine and transport in the circulatory system (Borel et al. 1996, Parker 1996, van den Berg 1999). In birds, there is a positive relationship between the polarity of carotenoids and their concentration in the blood and yolks of chickens (Na et al. 2004). Lutein is more polar than  $\beta$ -carotene and  $\beta$ -cryptoxanthin (Fig. 1) and may have been more readily available for absorption than the other two carotenoids in Yellow-headed Blackbirds.

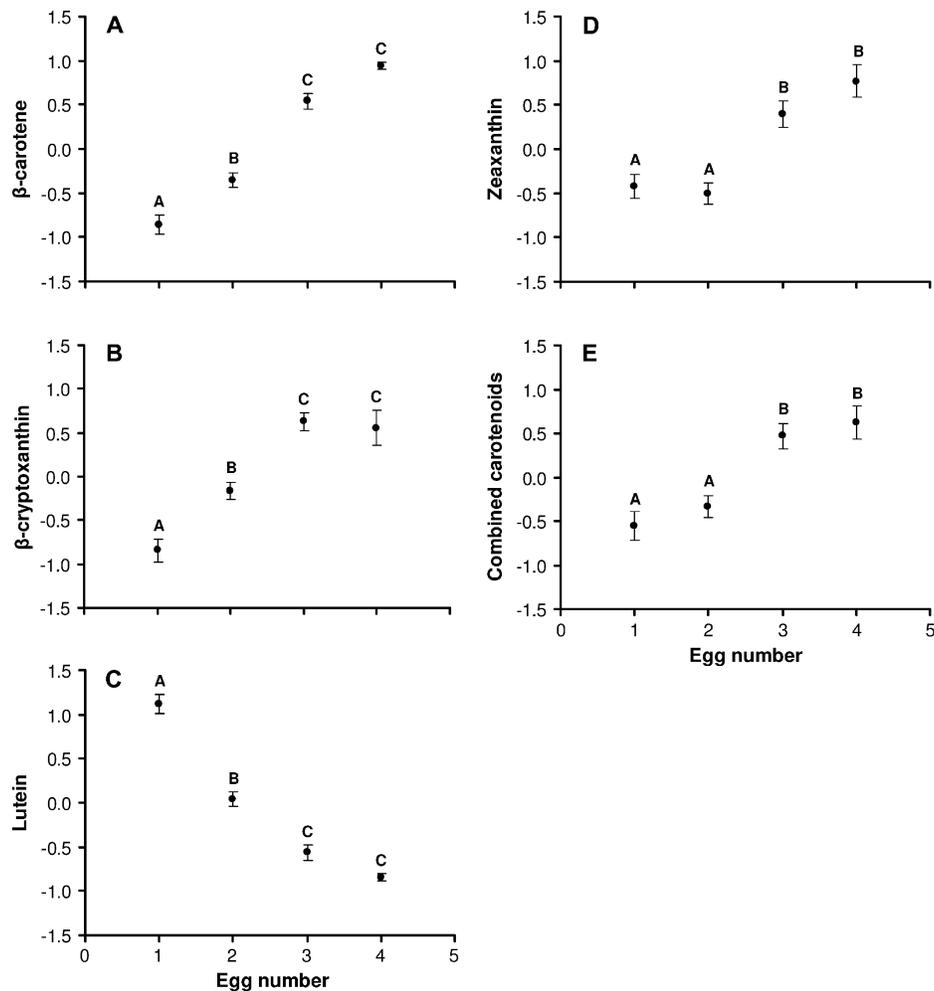


FIG. 2. Z-score standardized concentrations of (A)  $\beta$ -carotene, (B)  $\beta$ -cryptoxanthin, (C) lutein, (D) zeaxanthin, and (E) combined carotenoids in first- to fourth-laid Yellow-headed Blackbird eggs. Values are Z-score means  $\pm$  SE. Letters above values indicate results of Bonferroni *post-hoc* comparisons. Eggs with the same letter designations were not statistically distinguishable.

However, lutein and zeaxanthin have similar polarities and are expected to have similar properties affecting absorption, which suggests that other mechanisms may also have contributed to the carotenoid concentration patterns we observed.

Although the mechanisms of carotenoid allocation to eggs are unknown, differences in function among the specific carotenoids suggest that the patterns of allocation may be adaptive. All carotenoids act as antioxidants in bird eggs, but under laboratory conditions, carotenoids vary in their ability to react with free radicals (Edge et al. 1997). Martin et al. (1999) found an increase in carotenoid antioxidant ability with increasing number of functional groups. On the basis of these findings, lutein and zeaxanthin should function as better antioxidants in Yellow-headed Blackbird eggs than  $\beta$ -cryptoxanthin and  $\beta$ -carotene. Carotenoids also function as enhancers of the immune response by stimulating both cell-mediated and humoral immunity (Blount et al. 2000). Barn Swallow eggs with experimentally elevated levels of lutein produced chicks with stronger immune responses and

with higher fledging success than chicks from control eggs (Saino et al. 2003). Recent research with mammals has shown that lutein is more active in enhancing the immune response than  $\beta$ -carotene (Chew and Park 2004), but further research is needed to determine whether there are similar carotenoid-specific immune-enhancing differences in bird eggs.

The four yolk carotenoids we quantified showed different patterns of variation across the laying sequence. On the basis of our results, we caution that using one representative carotenoid to study laying-sequence variation in carotenoid allocation to eggs may provide misleading results. For example, if we had quantified only lutein, we would have concluded that female Yellow-headed Blackbirds allocate the most carotenoids to their first-laid eggs; but if we had quantified only  $\beta$ -carotene, we would have concluded the opposite. If representative carotenoids are used in future studies, careful consideration should be made when selecting them (i.e., selecting the most prevalent or the one with the most critical biological function) to avoid making erroneous or

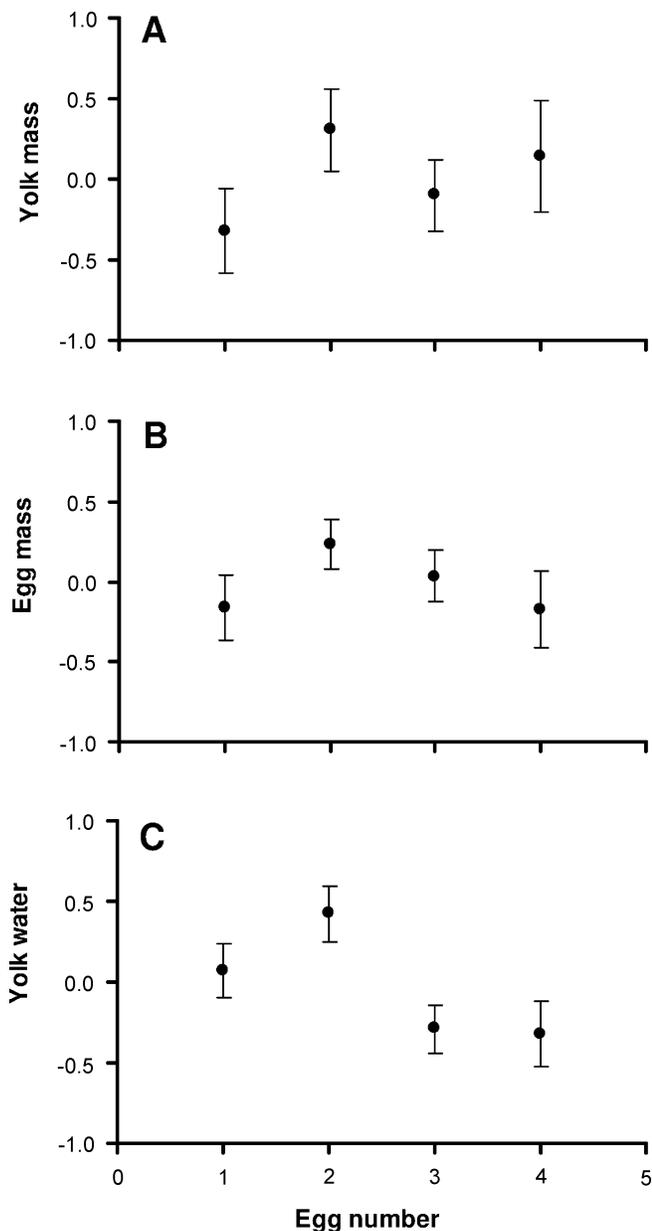


FIG. 3. Z-score standardized values of (A) egg mass, (B) yolk mass, and (C) yolk water content in first- to fourth-laid Yellow-headed Blackbird eggs. Values are z-score means  $\pm$  SE. There were no significant differences in any of the egg metrics with laying sequence, but general trends were observed.

incomplete conclusions. We also recommend that future studies provide data on laying-sequence variation in concentrations of both total and individual carotenoids to help increase our understanding of carotenoid interactions during absorption and potential differences in carotenoid functions in the egg yolk. Other maternal effects, such as androgens and vitamin antioxidants, may also covary with carotenoid deposition across the laying sequence.

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