



## YOLK AND FEATHER CAROTENOIDS IN RELATION TO FEMALE CONDITION AND REPRODUCTION IN THE YELLOW-HEADED BLACKBIRD (*XANTHOCEPHALUS XANTHOCEPHALUS*)

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**ABSTRACT.**—Carotenoids can be important antioxidants and immunostimulants for developing embryos and adult birds, and they are often incorporated into colorful sexual displays as signals of individual quality. The allocation of carotenoids to egg yolks and feathers can be affected by a female's physical condition, which can in turn affect offspring growth and survival. We examined relationships between yolk and feather concentrations of carotenoids and various indices of female quality (i.e., body condition, heterophil-to-lymphocyte ratio, hematocrit, date of nest initiation, egg mass, reproductive success) in the Yellow-headed Blackbird (*Xanthocephalus xanthocephalus*), a brightly colored passerine. Measures of physical condition of female Yellow-headed Blackbirds were correlated with the allocation of carotenoids to both eggs and feathers. Specifically, less stressed (i.e., lower heterophil-to-lymphocyte ratio) females had higher concentrations of  $\beta$ -carotene in their eggs than females that were more stressed. In addition, females with higher hematocrit values (i.e., more red blood cells) had higher concentrations of feather carotenoids. Timing of breeding was also related to variation in yolk carotenoid concentrations; later-breeding females produced eggs with lower concentrations of  $\beta$ -carotene but higher concentrations of lutein, which could be due to renesting or seasonal differences in carotenoid availability and use. Concentrations of yolk and feather carotenoids were not correlated, which suggests that individual carotenoids may have different functions in eggs and plumage. Carotenoid concentrations and female condition variables were not significantly related to the reproductive traits measured, but small sample sizes of females that fledged young may have limited our ability to detect effects. Our results highlight the need for further studies on the condition-dependent allocation and use of carotenoid pigments for reproduction and sexual signals in brightly colored female birds. Received 17 September 2010, accepted 9 February 2011.

Key words: beta-carotene, hematocrit, heterophil-to-lymphocyte ratio, lutein, reproductive success, *Xanthocephalus xanthocephalus*.

### Vitellus et caroténoïdes des plumes en relation avec la condition physique et la reproduction de la femelle chez *Xanthocephalus xanthocephalus*

**RÉSUMÉ.**—Les caroténoïdes peuvent être d'importants antioxydants et immunostimulants pour les embryons en développement et les oiseaux adultes et ils sont souvent incorporés aux signaux visuels colorés servant d'indices de la qualité des individus. L'allocation des caroténoïdes au vitellus des œufs et aux plumes peut être affectée par la condition physique de la femelle, laquelle peut en retour affecter la croissance et la survie de la progéniture. Nous avons examiné les relations entre les concentrations en caroténoïdes dans le vitellus et les plumes et divers indices de la qualité de la femelle (condition physique, rapport hétérophiles/lymphocytes, hématocrite, date de début de la nidification, masse de l'œuf et succès reproductif) chez *Xanthocephalus xanthocephalus*, un passereau aux couleurs vives. Les mesures de la condition physique des femelles ont été corrélées avec l'allocation des caroténoïdes dans les œufs et les plumes. Les femelles les moins stressées (rapport hétérophiles/lymphocytes faible) avaient des concentrations de  $\beta$ -carotène plus élevées dans leurs œufs que les femelles plus stressées. De plus, les femelles ayant des valeurs d'hématocrites plus élevées (plus de globules rouges) avaient des concentrations en caroténoïdes plus importantes dans les plumes. La chronologie de la reproduction était également associée à la variation des concentrations en caroténoïdes vitellins. Les femelles se reproduisant plus tardivement ont produit des œufs avec des concentrations en  $\beta$ -carotène plus faibles et des concentrations en lutéine plus élevées, qui pourraient être attribuables à la renidification ou à des différences saisonnières de la disponibilité des caroténoïdes et de leur utilisation. Les concentrations en caroténoïdes du vitellus et des plumes n'étaient pas corrélées, ce qui suggère que les caroténoïdes individuels peuvent avoir des

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fonctions différentes dans les œufs et le plumage. Les concentrations en caroténoïdes et les variables de la condition de la femelle n'étaient pas significativement reliées aux paramètres reproductifs mesurés, mais la taille réduite de l'échantillon de femelles dont les jeunes ont atteint l'envol peut avoir limité notre capacité à détecter les effets. Nos résultats soulignent la nécessité de poursuivre des recherches sur l'allocation des caroténoïdes en fonction de la condition et l'utilisation de ces pigments pour la reproduction et les signaux sexuels chez les oiseaux femelles aux couleurs vives.

CAROTENOIDS ARE BIOLOGICALLY active yellow, orange, and red pigments synthesized by plants and photosynthetic microorganisms, which animals obtain from their diets (Blount et al. 2000). More than 600 carotenoids have been identified in nature, and many carry out diverse functions in animals, including essential roles as antioxidants, immunostimulants, and precursors to vitamin A (Goodwin 1984). Carotenoids are also incorporated into the colorful sexual signals of many animals, including crustaceans, insects, fish, and birds, where they are thought to honestly reflect individual quality (Blount et al. 2000, Møller et al. 2000, Faivre et al. 2001). It is believed that carotenoids are rare in nature and, therefore, that carotenoid-based sexual advertisements are costly for animals to produce (Olson and Owens 1998). Carotenoids are also utilized for somatic maintenance and immune defense (Blount et al. 2000), which can lead to condition-dependent tradeoffs between the use of carotenoids for physiological functions and the expression of sexual signals (Saino et al. 2002). Because of limited carotenoid availability or use, only animals in good physical condition can enhance their carotenoid-based ornamentation, because they require fewer carotenoids for self-maintenance (Blount et al. 2003).

In birds, carotenoids are found in high concentrations in the skin, feathers, adipose tissue, liver, ovaries, and egg yolk (Goodwin 1984). In the yolk, carotenoids protect vulnerable embryonic tissues against damage caused by free radicals. The latter are byproducts of normal metabolism and immune defense that may damage DNA, proteins, and lipids (Surai et al. 2001). Female birds in good physical condition may allocate a greater diversity and higher concentrations of carotenoids to their egg yolks than females in poor condition, which can affect offspring growth and survival (Saino et al. 2002). For example, the probability that hatchling Lesser Black-backed Gulls (*Larus fuscus*) survived the first few days of life increased with increasing egg carotenoid concentrations (Royle et al. 1999). Similarly, immune systems of nestling Blue Tits (*Parus caeruleus*) were stronger and developed faster as the concentration of carotenoids in eggs increased (Biard et al. 2005). Overall hatching and fledging success of Zebra Finches (*Taeniopygia guttata*) also increased as the concentrations of carotenoids in eggs increased (McGraw et al. 2005).

In addition to carotenoids, the physical condition of a female bird, which is often quantified using size-corrected body mass (see below for further description), can also affect avian reproduction by influencing egg size, timing of breeding, and the number and quality of young. The importance of egg size for hatching and nestling performance has long been recognized (Williams 1994, Krist 2011), and egg size has been shown to be positively correlated with female condition in many bird species (Christians 2002). Large eggs often produce hatchlings that are more likely to survive (Williams 1994, Krist 2011), and in many passerine species, larger eggs produce heavier hatchlings that have greater nutrient reserves and greater survival probabilities than hatchlings from smaller eggs

(e.g., Smith et al. 1995, Styrsky et al. 2000, Pinowska et al. 2004, Rutkowska and Cichoń 2005). Female birds in good physical condition also tend to initiate breeding earlier in the season, lay larger clutches, and fledge more chicks than females in poor condition (Nooker et al. 2005). For example, female Ural Owls (*Strix uralensis*) in good condition laid earlier and larger clutches than those in poor condition (Pietiäinen and Kolunen 1993), and nest initiation date was earlier among females in good condition in three species of ducks (Blums et al. 2005). Also, female Tree Swallows (*Tachycineta bicolor*) in better condition produced heavier nestlings than those with experimentally reduced condition (Nooker et al. 2005), and egg size and hatchability were positively correlated with female condition in the Eastern Kingbird (*Tyrannus tyrannus*; Murphy 2004) and Barn Swallow (*Hirundo rustica*; Saino et al. 2004). Female condition and avian egg size have also been found to be positively correlated with yolk carotenoid concentrations. For example, female Upland Geese (*Chloephaga picta leucoptera*) in better condition produced larger eggs with higher carotenoid concentrations than females in poor condition (Gladbach et al. 2010), and egg size was positively correlated with yolk carotenoid concentrations and chick size and growth rates in the Yellow-legged Gull (*Larus michahellis*; Saino et al. 2008).

The overall physical condition of a bird is often determined by quantifying body condition, stress level, immune status, or a combination of these measures. Avian body condition is typically quantified using the mass of an individual corrected by size to provide an index of an individual's energy reserves (Pietiäinen and Kolunen 1993, Blums et al. 2005). Other noninvasive methods used to quantify adult body condition include scoring visible subcutaneous fat, feather development (Brown 1996), and pectoral muscle size (Gosler and Harper 2000). However, when compared with other methods, size-corrected body mass has been found to provide accurate information on stored energy reserves (DeLong and Gessaman 2001). Hematocrit (i.e., the volume of packed red blood cells) is also commonly measured as an index to general health status and stress level; birds experiencing physiological stress often have a lower proportion of red blood cells than females that are less stressed (Clinchy et al. 2004). For example, birds stressed by a lack of food (Hoi-Leitner et al. 2001), exposed to high parasite loads (Merino and Potti 1998), or undergoing migration or molt without adequate fat reserves (Svensson and Merilä 1996) tend to have lower hematocrit values than birds exposed to less stressful conditions. However, hematocrit has been found to be consistent across years, which suggests that it is a useful trait for comparisons of physiological condition among individual birds (Hatch and Smith 2010). A third metric commonly measured to describe female physical condition is the heterophil-to-lymphocyte (H:L) ratio, which provides information on variation in stress levels and immune status (Gross and Siegel 1983, Norris and Evans 2000); birds exposed to high levels of stress, including infectious disease, starvation, and high levels of disturbance, tend to have higher H:L

ratios than less stressed individuals (Gross and Siegel 1983, Maxwell 1993, Ots et al. 1998, Clinchy et al. 2004). The physical condition of a female bird may also affect the allocation of carotenoids to eggs and plumage. For example, female House Finches (*Carduelis mexicanus*) in better condition allocated higher concentrations of carotenoids to their eggs than females in poorer condition (Navara et al. 2006), and female Upland Geese in better condition had brighter carotenoid-based plumage (Gladbach et al. 2010). However, the majority of avian studies on condition-dependent allocation of carotenoids to colorful plumage and reproduction have focused on male birds.

In the present study, we examined relationships between yolk and feather concentrations of carotenoids and various indices of female quality (i.e., body condition, H:L ratio, hematocrit, date of nest initiation, egg mass, reproductive success) in the Yellow-headed Blackbird (*Xanthocephalus xanthocephalus*). We predicted that females in good physical condition would have higher concentrations of carotenoids in yolks and feathers than females in poor condition, and that female Yellow-headed Blackbirds with higher concentrations of yolk and feather carotenoids would produce larger eggs and have better reproductive success than females with lower concentrations. We also evaluated correlations among concentrations of individual carotenoids between yolks and feathers of adult females. Although the use of limited carotenoid resources for many different functions can lead to tradeoffs (Biard et al. 2009), birds in good physical condition may be able to allocate more carotenoids to both eggs (Saino et al. 2002) and carotenoid-based ornamentation (Blount et al. 2003) because they require fewer of their limited carotenoid resources for self-maintenance and immune defense. Therefore, we predicted that female Yellow-headed Blackbirds with high concentrations of carotenoids in their feathers would also produce eggs with high concentrations of carotenoids in their yolks.

## METHODS

**Study site and study species.**—This study was conducted on five deep-water, palustrine wetlands in Stutsman County, North Dakota, from 15 May to 25 June 2005. Central North Dakota has abundant kettle lake basins and wetlands and one of the highest concentrations of breeding Yellow-headed Blackbirds in North America (Twedt and Crawford 1995). All study wetlands were small (<8 ha) and had extensive stands of emergent cattail (*Typha* spp.), which are preferred breeding sites for Yellow-headed Blackbirds.

The Yellow-headed Blackbird is a large passerine of the family Icteridae that is sexually dimorphic in both size and plumage. Males are nearly twice the average mass of a female (100 g vs. <60 g; Twedt and Crawford 1995). Both male and female Yellow-headed Blackbirds have yellow feathers on their head, throat, and breast, but the female's yellow plumage is much reduced compared with males. Adult Yellow-headed Blackbirds molt once a year, starting in late June and ending in September; juvenile Yellow-headed Blackbirds obtain their yellow plumage after they have completed a prebasic molt at ~80 days of age (Twedt and Crawford 1995).

Yellow-headed Blackbirds breed in colonies and males mate polygynously with one to six females in a male's territory. Females build open-cup nests, which are suspended at least 15 cm above the surface of water that is at least 30 cm deep (Twedt and Crawford

1995). They are determinant layers with a normal clutch size of 4 eggs, and individual females lay eggs that are highly consistent in both size (i.e., length and width) and volume, both within and across clutches (Arnold 1992, Newbrey 2007). Most females incubate, brood, and provision their nestlings without the aid of the territorial male, although some males on unproductive marshes will provide care to the nestlings of their primary female (Gori et al. 1996). Breeding Yellow-headed Blackbirds feed preferentially on odonates and other aquatic insects, but they also feed on invertebrates from upland habitats (Twedt and Crawford 1995).

**Field methods.**—Each wetland was searched daily for active Yellow-headed Blackbird nests. Only recently initiated nests with two or fewer eggs were included in the study. Global positioning system coordinates with 3-m accuracy (Magellan, San Dimas, California) were obtained for a total of 69 study nests, and numbered orange flagging-tape markers were tied to nearby vegetation for individual nest identification. The contents of each nest were recorded, and if eggs were present they were marked with small black nontoxic ink dots on the blunt end to indicate laying sequence. Nests were then monitored throughout the laying sequence, and the third-laid egg was collected on the day it was laid for measurement of egg characteristics and carotenoids. Although we did not measure the size of all eggs of a female's clutch, the third egg is a good representation of the size of the other eggs of a clutch in the species (Arnold 1992, Newbrey et al. 2008). Most carotenoids, with the exception of lutein, increase in concentration across the laying sequence in Yellow-headed Blackbird clutches, which results in third- and fourth-laid eggs having higher carotenoid concentrations than earlier-laid eggs (Newbrey et al. 2008). Intraspecific nest parasitism is extremely rare in the Yellow-headed Blackbird (Twedt and Crawford 1995), and only one egg was added to each nest daily, so we are confident that only one female laid eggs in each study nest. Collected eggs were kept on ice for ≤6 h before each egg was weighed to the nearest 0.1 g and the length and breadth measured to the nearest 0.1 mm. Measured eggs were then wrapped in Parafilm and frozen at -20°C until carotenoid analysis (see below).

The female of each study nest was captured during the incubation stage of the nesting cycle using a nest trap (Newbrey and Reed 2008) between 0900 and 1630 hours CDT. All birds were removed promptly from the trap to reduce stress associated with capture. Females were weighed to the nearest 0.5 g using a spring scale, and tarsus lengths were measured to the nearest 0.1 mm using a digital caliper. Three to five yellow feathers were collected from a standard position on the center of the throat to quantify carotenoid concentrations in feathers. Prior to release, each female was banded with a U.S. Geological Survey band and a small blood sample (<150 µL) was collected via venous puncture at the brachial vein. A drop of blood was smeared onto a microscope slide and allowed to air dry to create a blood smear for quantification of blood leukocytes. The remaining whole blood was kept in heparinized microcapillary tubes on ice for ≤6 h until lab analysis of hematocrit.

Study nests were visited daily to determine the number of eggs laid, the number of eggs hatched, and the number of nestlings fledged. All nestlings that survived to 8 days of age were considered to have fledged, because female nestlings in the study area fledge at 8–10 days and males fledge at 9–11 days.

**Laboratory methods.**—Microcapillary tubes containing blood samples were centrifuged for 5 min at  $6,000 \times g$ , and the proportion of plasma and packed red blood cells was measured to the nearest 1 mm with a ruler. The packed-red-blood-cell volume was divided by the total blood volume for three to six tubes for each female to obtain the mean percent cellular fraction (hematocrit). Across-tube variation was very low for individual females ( $CV = 0.7\%$ ). Blood smears were fixed in methanol and stained using a Hemacolor stain set (EMD Chemicals, Gibbstown, New Jersey). One hundred white blood cells were identified as lymphocytes or heterophils using a  $1,000\times$  oil immersion lens, and the ratio of the two cell types (H:L ratio) was calculated for each female (Vleck et al. 2000). The process was repeated for an additional 100 white blood cells, and an average H:L ratio was calculated for each female and used in all analyses described below. Across-count variation was low for individual females ( $CV = 9.9\%$ ).

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) of homogenized yolk 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). This method of drying does not influence the carotenoid content of yolk samples (Newbrey et al. 2008). 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) was added to each sample.

The method used to extract carotenoids from yolks was modified from Surai et al. (2001). In short,  $500 \mu\text{L}$  5% NaCl(aq) solution was added to a yolk sample, vortexed for 2 min,  $1 \text{ mL}$  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  $2\text{-}\mu\text{m}$  syringe filters (National Scientific, Rockwood, Tennessee).

Carotenoid extraction from feathers was modified from McGraw et al. (2002). The feathers were washed in ethanol, then hexane, and blotted dry on filter paper. The distal yellow portion of each feather was removed and weighed to the nearest 0.01 mg. The yellow feather samples (1–2 mg) were then placed into 8-mL glass tubes,  $1 \text{ mL}$  of acidified pyridine was added, and the headspace was flushed with nitrogen. The samples were incubated at  $95^\circ\text{C}$  in a covered waterbath for 3 h. After cooling to room temperature,  $0.365 \mu\text{g}$  of internal standard (astaxanthin in  $2 \mu\text{L}$  dichloromethane) and  $1 \text{ mL}$  of water were added, and the samples were vortexed for 1 min. Next,  $1.5 \mu\text{L}$  of *tert*-butyl methyl ether was added, the samples were vortexed for 2 min, and then centrifuged at  $1,300 \times g$  for 5 min. Samples were frozen in an ethanol bath cooled with dry ice, and the unfrozen upper organic layer, containing the carotenoids, was decanted. The samples were dried under nitrogen, resuspended in  $150 \mu\text{L}$  of the mobile phase (see below), filtered, and transferred into  $2\text{-mL}$  vials. Although feather samples were taken from each captured female, 30 individuals did not have adequate yellow feather mass (i.e., mass  $< 1 \text{ mg}$ ) for carotenoid extraction and were therefore excluded from all feather analyses described below.

Twenty microliters of each carotenoid extract from yolks and feathers was injected into a Shimadzu SCL-6A high-performance liquid chromatography (HPLC) system fitted with a normal phase silica column ( $100 \text{ }\mu\text{m}$ ;  $250 \times 4.6 \text{ mm}$ ; 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 in yolk and feather extracts 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 calculate concentrations of carotenoids in yolk and feather samples, calibration curves were created for each individual carotenoid and the internal standard.

The amount of variation associated with our yolk carotenoid extraction and quantification methods was previously found to be low for Yellow-headed Blackbird egg yolk carotenoids:  $\beta$ -carotene ( $CV = 8.8\%$ ),  $\beta$ -cryptoxanthin ( $CV = 8.5\%$ ), lutein ( $CV = 8.7\%$ ), and zeaxanthin ( $CV = 9.3\%$ ) (Newbrey et al. 2008). To determine the amount of variation associated with our feather-carotenoid extraction and quantification methods, we extracted and quantified carotenoids from six replicates of five yellow feathers from the breast of a male Yellow-headed Blackbird. Variation in the concentrations of individual feather carotenoids ranged from 9% to 16%:  $\beta$ -carotene ( $CV = 10.4\%$ ),  $\beta$ -cryptoxanthin ( $CV = 8.7\%$ ), lutein ( $CV = 9.2\%$ ), and zeaxanthin ( $CV = 16.3\%$ ).

**Statistical analyses.**—Mass was regressed against tarsus length ( $r^2 = 0.104$ ,  $P = 0.007$ ), and the residuals were used as a measure of maternal body condition in all analyses.

Pearson correlation matrices were created to identify highly correlated variables. Concentrations of individual carotenoids were not correlated between yolk and feather samples ( $r = -0.295$  to  $0.036$ ,  $P > 0.990$  for all). Yolk concentrations of  $\beta$ -carotene,  $\beta$ -cryptoxanthin, and zeaxanthin were all positively correlated with one another ( $r = 0.793$ – $0.944$ ,  $P < 0.001$ ). Yolk concentrations of lutein were weakly and negatively correlated with concentrations of the other three yolk carotenoids ( $r = -0.470$  to  $-0.418$ ,  $P = 0.161$ – $0.431$ ). Based on the results of the correlation matrices, two representative yolk carotenoids were used in all analyses described below,  $\beta$ -carotene and lutein. We selected  $\beta$ -carotene because it is the most common carotenoid in egg yolks, and lutein because it exhibits unique intraclutch variation patterns when compared with other carotenoids in Yellow-headed Blackbird eggs (Newbrey et al. 2008). Concentrations of all individual feather carotenoids were weakly and positively correlated with one another ( $r = 0.473$ – $0.784$ ,  $P = 0.306$ – $0.001$ ), and therefore total feather carotenoids were used for all analyses. None of the three female condition parameters were correlated with one another ( $r = -0.029$  to  $0.236$ ,  $P = 0.157$ – $1.000$ ).

Linear regression was used to describe the relationships between female characteristics, yolk  $\beta$ -carotene, yolk lutein, feather carotenoid concentrations, and egg mass. Because the data were non-normally distributed, yolk lutein and total feather carotenoid concentrations were natural log transformed and H:L ratio was normalized using a square-root transformation prior to all analyses.



Akaike's information criterion corrected for small sample size ( $AIC_c$ ; Burnham and Anderson 1998) was used to select the best linear model of female quality parameters that described variation in carotenoid concentrations.  $AIC_c$  is a second-order variant of AIC, which is used when there are many parameters in relation to the size of the sample (Burnham and Anderson 1998). We considered all models with an  $AIC_c$  within 10 units of the best model to be viable alternative models. However, we considered models with an  $AIC_c$  within 2 or between 3 and 7 units of the best model to provide substantial and less support, respectively (Burnham and Anderson 2001). Akaike weights ( $w_i$ ), which represent the relative likelihood that a specific model is the best of a suite of models (Burnham and Anderson 1998), were also calculated for each candidate model.

Females that fledged at least one chick were grouped into categories based on the number of eggs hatched (i.e., 1–4) and number of chicks fledged (i.e., 1–3), and carotenoid concentrations and female traits were compared using general linear models (GLMs) with number of eggs laid and number of eggs hatched as covariates in the models, respectively. Because of the large number of tests run, GLMs were considered significant at an adjusted alpha value ( $P \leq 0.007$ ), which was calculated using a Holm simultaneous testing procedure (Neter et al. 1996). Alpha was adjusted for each family of test that shared the same factor, and the most conservative alpha value was selected to reduce the chances of making a Type I error (Neter et al. 1996).

Females were also categorized as either early-breeding or late-breeding (early-breeding = Julian day  $\leq 145$ ,  $\bar{x} = 140.2$ ,  $n = 55$ ; late-breeding = Julian day  $> 145$ ,  $\bar{x} = 159.8$ ,  $n = 14$ ) on the basis of a bimodal distribution in nest initiation dates (i.e., date of first egg). Female characteristics and carotenoid concentrations were then compared using two-sample  $t$ -tests with  $\alpha = 0.05$ . All analyses were performed using the statistical program SYSTAT, version 11 (Systat Software, Richmond, California).

## RESULTS

Carotenoid concentrations in yolks and feathers were more variable among female Yellow-headed Blackbirds than were our measures of female condition and reproduction (Table 1). The same

four carotenoids were identified in feathers of female Yellow-headed Blackbirds as were previously described from their yolks (Newbrey et al. 2008):  $\beta$ -carotene,  $\beta$ -cryptoxanthin, lutein, and zeaxanthin (Fig. 1). Lutein was the most abundant carotenoid in the feathers of female Yellow-headed Blackbirds and represented, on average, 64.5% of identified carotenoids ( $\bar{x} = 49.70 \mu\text{g g}^{-1}$  feather). The next most prevalent feather carotenoid was zeaxanthin (17.0%,  $\bar{x} = 12.53 \mu\text{g g}^{-1}$  feather), followed by  $\beta$ -cryptoxanthin (11.7%,  $\bar{x} = 8.65 \mu\text{g g}^{-1}$  feather) and  $\beta$ -carotene (6.8%,  $\bar{x} = 4.55 \mu\text{g g}^{-1}$  feather). The carotenoids in egg yolks showed the exact opposite trend, with the most abundant carotenoid being  $\beta$ -carotene (37.4%,  $\bar{x} = 66.69 \mu\text{g g}^{-1}$  yolk), followed by  $\beta$ -cryptoxanthin (32.4%,  $\bar{x} = 59.11 \mu\text{g g}^{-1}$  yolk), zeaxanthin (17.7%,  $\bar{x} = 30.66 \mu\text{g g}^{-1}$  yolk), and lutein (12.5%,  $\bar{x} = 19.34 \mu\text{g g}^{-1}$  yolk).

Linear regression analyses revealed that female body condition was not significantly related to time of day at capture ( $r^2 = 0.016$ ,  $P = 0.307$ ) or date of capture ( $r^2 = 0.055$ ,  $P = 0.053$ ), although the negative relationship between female condition and date of capture was nearly significant. Also, egg mass was not significantly related to any of the carotenoid concentrations (yolk  $\beta$ -carotene,  $r^2 = 0.005$ ,  $P = 0.562$ ; ln [yolk lutein],  $r^2 = 0.002$ ,  $P = 0.729$ ; ln [feather carotenoids],  $r^2 = 0.011$ ,  $P = 0.536$ ), body condition ( $r^2 = 0.003$ ,  $P = 0.950$ ), H:L ratio (square root [H:L],  $r^2 = 0.000$ ,  $P = 0.916$ ), hematocrit ( $r^2 = 0.017$ ,  $P = 0.302$ ), or nest initiation date ( $r^2 = 0.013$ ,  $P = 0.365$ ).

According to the results of model selection, variation in yolk  $\beta$ -carotene concentrations was best explained by H:L ratio and variation in yolk lutein concentration was best explained by body condition, although hematocrit also had support from the lutein data (Table 2). Variation in feather carotenoid concentrations was explained best by hematocrit, but an association with body condition and H:L ratio also had support (Table 2).

All of the GLMs that compared carotenoid concentrations and female traits for females grouped into categories based on the number of eggs hatched and number of chicks fledged were nonsignificant (Table 3). However, there were nearly significant differences in yolk  $\beta$ -carotene and yolk lutein concentrations between female Yellow-headed Blackbirds that laid clutches of 3 and 4 eggs (Fig. 2). Early-breeding female Yellow-headed Blackbirds had higher concentrations of  $\beta$ -carotene in their yolks ( $t = 8.624$ ,

TABLE 1. Variables measured to study maternal condition, carotenoid allocation, and reproduction in Yellow-headed Blackbirds in North Dakota in 2005.

Variable	<i>n</i>	Mean $\pm$ SD	Coefficient of variation	Range
Body condition <sup>a</sup>	69	0.00 $\pm$ 3.11	4.51%	–6.68 to 7.66
H:L ratio <sup>b</sup>	69	0.24 $\pm$ 0.17	0.24%	0.01 to 0.91
Hematocrit (% cellular fraction)	68	50.15 $\pm$ 3.53	5.19%	41.69 to 58.14
Yolk $\beta$ -carotene ( $\mu\text{g g}^{-1}$ yolk)	67	66.69 $\pm$ 22.02	32.86%	19.21 to 111.08
Yolk lutein ( $\mu\text{g g}^{-1}$ yolk)	66	19.34 $\pm$ 11.56	17.52%	5.29 to 58.35
Feather carotenoids ( $\mu\text{g g}^{-1}$ feather)	39	79.00 $\pm$ 41.13	105.85%	20.48 to 158.87
Egg mass (g)	66	4.50 $\pm$ 0.30	0.46%	3.80 to 5.10
Number of eggs laid	69	3.93 $\pm$ 0.36	0.52%	3 to 5
Number of eggs hatched	59	2.29 $\pm$ 0.97	1.54%	0 to 4
Number of chicks fledged	59	1.26 $\pm$ 1.09	1.92%	0 to 3

<sup>a</sup>Body condition = mass-tarsus residuals.

<sup>b</sup>H:L ratio = heterophil-to-lymphocyte ratio.

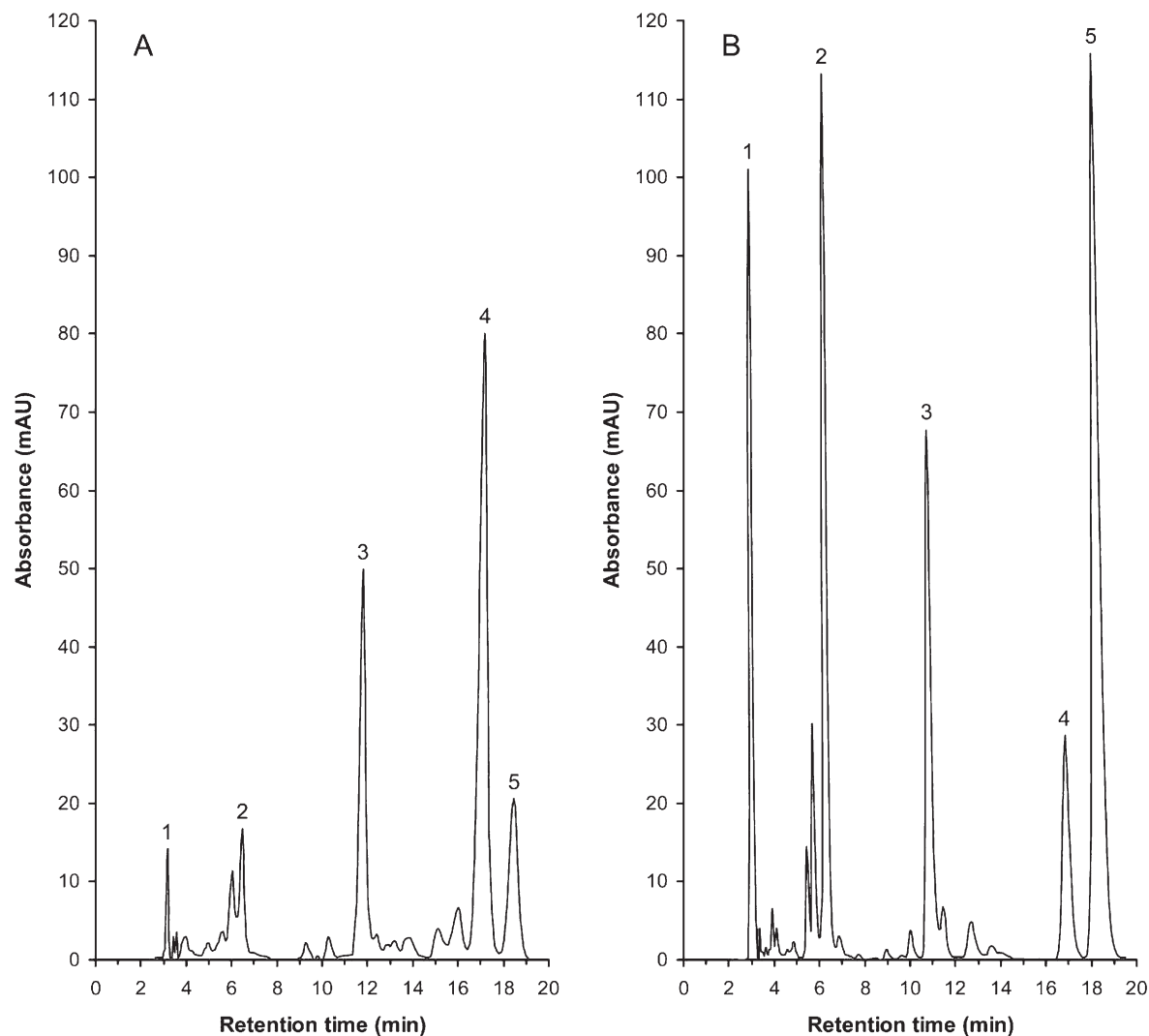


FIG. 1. High-performance-liquid-chromatography chromatograms of carotenoids found in typical female Yellow-headed Blackbird (A) yellow-colored throat feathers and (B) egg yolks in North Dakota in 2005. Separation was done using normal phase with detection at 450 nm. Peak 1 =  $\beta$ -carotene, peak 2 =  $\beta$ -cryptoxanthin, peak 3 = astaxanthin (internal standard), peak 4 = lutein, and peak 5 = zeaxanthin.

TABLE 2. Model selection results of single-variable models of female Yellow-headed Blackbird condition variables in relation to carotenoid concentrations in a North Dakota population in 2005. Models are ranked by Akaike's information criterion corrected for small sample sizes ( $AIC_c$ ). All models with  $\Delta AIC_c \leq 7$  are reported, and all models with  $\Delta AIC_c \leq 2$  were considered to have substantial support from the data.

Carotenoid	Model <sup>a</sup>	+/- <sup>b</sup>	Log likelihood	$AIC_c$	$\Delta AIC_c$	$w_i$
Yolk $\beta$ -carotene	Square root (H:L ratio)	–	–147.55	299.23	0	0.92
	Body condition	+	–149.98	304.08	4.86	0.08
Ln (yolk lutein)	Body condition	–	–41.19	86.51	0	0.52
	Hematocrit	–	–41.75	87.62	1.11	0.30
	Square root (H:L ratio)	+	–42.20	88.52	2.01	0.19
Ln (feather carotenoids)	Hematocrit	+	–24.72	53.65	0	0.55
	Square root (H:L ratio)	–	–25.60	55.43	1.77	0.23
	Body condition	+	–25.61	55.44	1.78	0.23

<sup>a</sup>Body condition = mass-tarsus residuals, H:L ratio = heterophil-to-lymphocyte ratio.

<sup>b</sup>+/- = positive or negative relationship.

TABLE 3. Results of general linear models of yolk and feather carotenoid concentrations ( $\mu\text{g g}^{-1}$ ) and female Yellow-headed Blackbird parameters with number of eggs hatched and number of nestlings fledged as factors, and number of eggs laid and number of eggs hatched as covariates, respectively. Alpha was adjusted using a Holm simultaneous testing procedure ( $P < 0.007$ ) because of the large number of tests run.

Factor	Covariate	Dependent variable <sup>a</sup>	Df	$r^2$	$F$		$P$	
					Factor	Covariate	Factor	Covariate
Number of eggs hatched	Number of eggs laid	Yolk $\beta$ -carotene	3, 1, 32	0.158	1.790	4.080	0.169	0.052
		Ln (yolk lutein)	3, 1, 31	0.196	1.598	3.411	0.210	0.074
		Ln (feather carotenoids)	3, 0, 14	0.098	0.504	—	0.685	—
		Body condition	1, 2, 34	0.055	0.062	0.876	0.804	0.426
		Square root (H:L ratio)	3, 1, 33	0.007	0.080	0.021	0.970	0.887
		Hematocrit	1, 2, 33	0.017	0.000	0.284	0.989	0.755
Number of fledglings	Number of eggs hatched	Yolk $\beta$ -carotene	2, 1, 33	0.021	0.126	0.620	0.882	0.437
		Ln (yolk lutein)	1, 3, 31	0.111	0.135	1.283	0.716	0.297
		Ln (feather carotenoids)	1, 3, 13	0.236	2.355	0.563	0.149	0.649
		Body condition	2, 1, 34	0.009	0.049	0.060	0.952	0.808
		Square root (H:L ratio)	1, 3, 33	0.014	0.230	0.087	0.634	0.966
		Hematocrit	2, 1, 33	0.029	0.498	0.046	0.612	0.832

<sup>a</sup>Body condition = mass-tarsus residuals, and H:L ratio = heterophil-to-lymphocyte ratio.

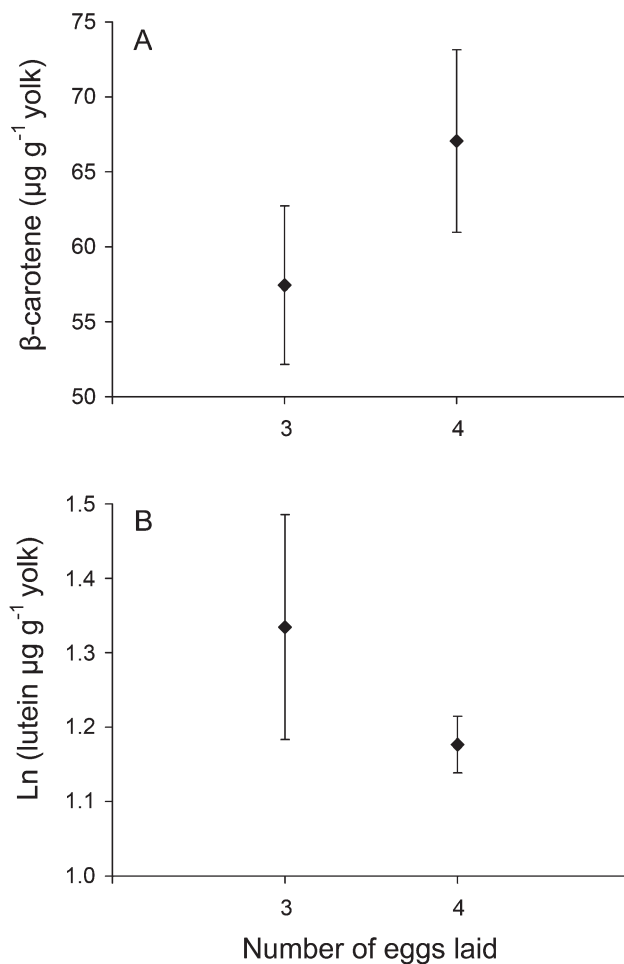


FIG. 2. Variation in (A) yolk  $\beta$ -carotene concentrations and (B) yolk lutein concentrations between female Yellow-headed Blackbirds that laid 3 and 4 egg clutches in North Dakota in 2005.

$df = 25.4$ ,  $P < 0.001$ ; Fig. 3A) and higher hematocrit ( $t = 2.194$ ,  $df = 25.7$ ,  $P = 0.037$ ; Fig. 3B) than late-breeding females. By contrast, early-breeding females had lower concentrations of yolk lutein ( $t = -2.220$ ,  $df = 17.8$ ,  $P = 0.040$ ), and nearly significantly lower H:L ratios ( $t = -2.072$ ,  $df = 15.4$ ,  $P = 0.055$ ), than late-breeding females. Female body condition ( $t = 1.848$ ,  $df = 22.1$ ,  $P = 0.078$ ), feather carotenoid concentrations ( $t = 1.304$ ,  $df = 10.6$ ,  $P = 0.220$ ), and egg mass ( $t = -1.135$ ,  $df = 21.9$ ,  $P = 0.269$ ) did not differ significantly between early- and late-breeding females.

## DISCUSSION

The feathers and egg yolks of female Yellow-headed Blackbirds contain the same four yellow carotenoid pigments, but in different proportions. Yellow-headed Blackbirds produce yolks with high concentrations of  $\beta$ -carotene and  $\beta$ -cryptoxanthin and lower concentrations of zeaxanthin and lutein, which is the opposite of all other passerines studied to date (e.g., Biard et al. 2005, Cassey et al. 2005, Ewen et al. 2006, Hargitai et al. 2006). By contrast, the most abundant carotenoid in the feathers of female Yellow-headed Blackbirds is lutein, followed by zeaxanthin,  $\beta$ -cryptoxanthin, and  $\beta$ -carotene. Lutein, which is the carotenoid most commonly found in the feathers of many other bird species, was found in concentrations roughly 4–11 $\times$  that of any other carotenoid present in Yellow-headed Blackbird feathers. Several North American passerines, including the Yellow Warbler (*Dendroica petechia*), Common Yellowthroat (*Geothlypis trichas*), and Evening Grosbeak (*Coccothraustes vespertinus*), pigment their feathers exclusively with lutein, even though both lutein and zeaxanthin have been found in their plasma; it is thought that these passerines selectively incorporate lutein into their feathers over other carotenoids (McGraw et al. 2003). The different carotenoid patterns observed between the yolks and feathers of female Yellow-headed Blackbirds could indicate that females are differentially allocating specific carotenoids to reproduction and colorful signals (i.e., lutein to feathers and  $\beta$ -carotene to egg yolks), or that individual

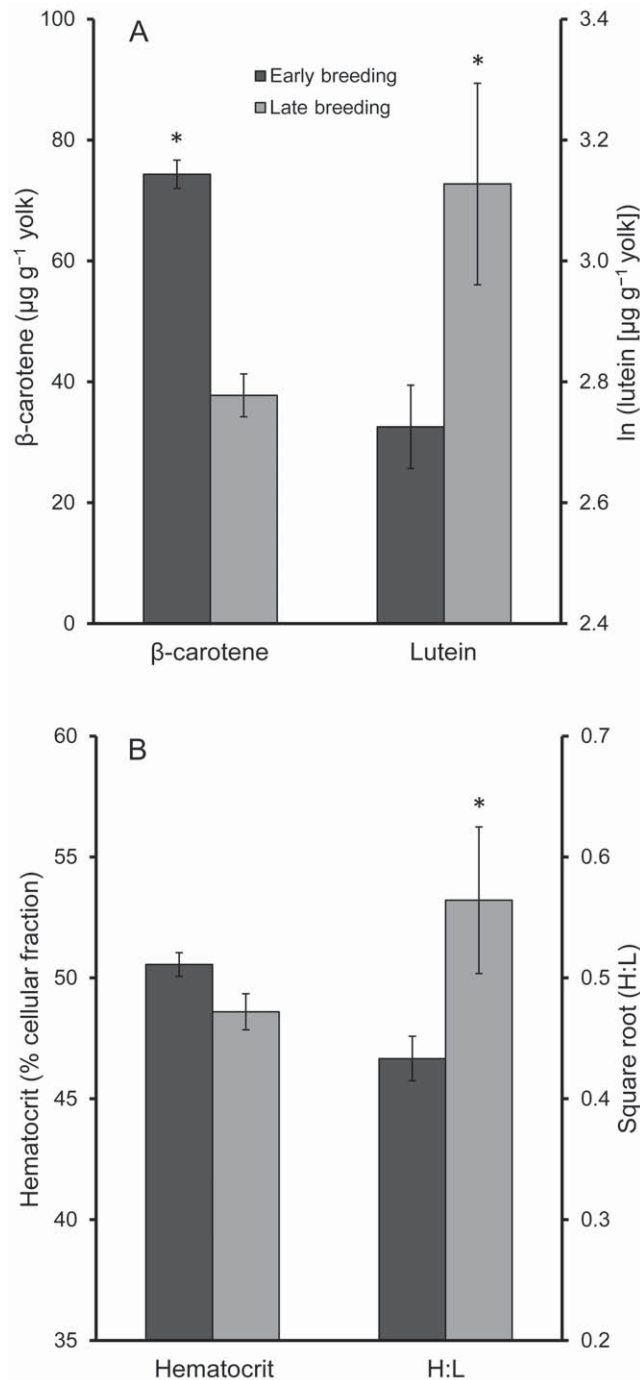


FIG. 3. Differences in (A) yolk carotenoid concentrations and (B) variables that describe female condition for early- and late-breeding female Yellow-headed Blackbirds in North Dakota in 2005. Error bars represent means  $\pm$  SE. Asterisk = significantly higher. H:L = heterophil-to-lymphocyte ratio.

carotenoid pigments may be differentially available during egg production versus molt (see below), but further research is needed to test these hypotheses.

Two condition measures of female Yellow-headed Blackbirds, H:L ratio and hematocrit, were significantly related to carotenoid

concentrations in yolks and feathers. Females with lower H:L ratios produced eggs with higher concentrations of  $\beta$ -carotene than females with higher ratios. Also, females with high hematocrit deposited more carotenoids in their feathers. These correlations between female Yellow-headed Blackbird traits and carotenoid concentrations suggest that females in better health and under less stress produced eggs and feathers with higher concentrations of carotenoids than females that were more stressed and in poorer health. Our results corroborate results reported in other studies of birds; for example, female Barn Swallows with unchallenged immune systems allocated higher concentrations of carotenoids to their yolks than females with experimentally challenged immune systems, which suggests that female birds have a limited supply of carotenoids needed for both physiological maintenance and reproduction (Saino et al. 2002). Also, female Upland Geese in better condition had brighter carotenoid-based plumage than females in poorer condition (Gladbach et al. 2010).

Contrary to our prediction that female Yellow-headed Blackbirds with high concentrations of carotenoids in their feathers would also produce eggs with high concentrations of carotenoids in their yolks, individual carotenoid concentrations of yolk and feathers were not related. Similar results were found in a population of Blue Tits and were attributed to differences in the availability of carotenoids during molt and reproduction, and also to potential tradeoffs in allocation and use of carotenoids for different functions (Biard et al. 2005). Annual and seasonal differences in the availability of carotenoids may also exist for female Yellow-headed Blackbirds, which could influence carotenoid allocation to yolks and feathers. In our study, most female Yellow-headed Blackbirds laid eggs in May and early June 2005, but molt occurs annually in late summer, so the feathers that we sampled were produced in late June to September 2004. As carotenoid availability in bird diets can vary greatly between seasons (Hargitai et al. 2006), our results suggest that carotenoid concentrations in a female Yellow-headed Blackbird's plumage are a poor predictor of carotenoid concentrations in the eggs she produces in the following year.

Early-breeding female Yellow-headed Blackbirds produced eggs with higher concentrations of  $\beta$ -carotene than late-breeding females, and they had higher hematocrit values and lower H:L ratios. These data suggest that early-breeding females were in better physiological condition than those that bred later (Fig. 3). Perhaps females with fewer carotenoid resources and in poor health delayed nest initiation, or later-nesting females were producing replacement clutches after their first nest attempts failed. Yellow-headed Blackbirds produce only one brood per season, but females will renest if their first nest attempt fails early in the breeding season (Twedt and Crawford 1995). Similar to other populations of Yellow-headed Blackbirds (i.e., Picman and Isabelle 1995), 59% of our study nests were initiated within a 5-day period, and 81% within 8 days. Later-breeding females (i.e., Julian day >145) accounted for only 20% of the females that we studied, which is a reasonable number to attribute to renesting. Renesting females likely had fewer carotenoid resources available for their second clutch because carotenoids are scarce in nature and females deplete their carotenoid stores as they lay more eggs (Saino et al. 2002). A limited availability of carotenoids has even been found to constrain the re-laying capacity of some female birds (Blount et al. 2004). Female Yellow-headed Blackbirds that bred later also had



lower hematocrit values and higher H:L ratios, possibly because of the physiological stress associated with producing a second clutch of eggs.

In contrast to the other carotenoids measured, females with low hematocrit values that bred later had higher concentrations of lutein in their yolks. We propose that the shift to lutein reflects seasonal differences in dietary lutein availability for female Yellow-headed Blackbirds. Lutein is the only carotenoid that decreases across the laying sequence in Yellow-headed Blackbird clutches, which suggests that it may be more limited in the diet than other carotenoids during egg production (Newbrey et al. 2008). Lutein is also the main carotenoid found in the feathers of Yellow-headed Blackbirds, which suggests that dietary items with high concentrations of lutein may be more common later in the breeding season, when females molt.

Differences in function of carotenoids suggest that the patterns of carotenoid allocation to yolks and feathers may be adaptive for female Yellow-headed Blackbirds. All carotenoids can act as antioxidants in bird eggs, but under laboratory conditions carotenoids vary in their ability to react with free radicals (Edge et al. 1997). For example,  $\beta$ -carotene did not reduce the number of free radicals as well as other carotenoids when tested in low-oxygen conditions similar to those found in embryonic tissues (Edge et al. 1997). Also, the antioxidant ability of carotenoids tends to increase with an increase in the number of functional groups (Martin et al. 1999). Therefore, lutein likely functions as a better antioxidant in Yellow-headed Blackbird eggs than  $\beta$ -carotene, and females may selectively incorporate lutein into their yolks as lutein becomes available later in the season.

We predicted that female Yellow-headed Blackbirds in good physical condition and those with higher carotenoid concentrations would hatch more eggs and fledge more chicks than females in poor condition. However, none of the female traits or the carotenoid concentrations in yolks or feathers varied significantly with the number of eggs hatched or the number of chicks fledged. Regardless of female health, many factors contribute to variation in reproductive success of females, and the ~40% of nests that failed because of nest predation and weather severely limited our ability to evaluate relationships among measures of female condition, yolk carotenoid concentrations, and reproductive success. However, elsewhere (Newbrey and Reed 2009) we have shown that yolk carotenoid concentrations were positively correlated with Yellow-headed Blackbird nestling mass and growth rates, and other studies have found positive associations between yolk carotenoids and nestling health and survival (e.g., Royle et al. 1999, Saino et al. 2003, Biard et al. 2005, McGraw et al. 2005). Also, our GLMs in the present study lacked strong statistical power (<50%) because of the small sample sizes of females that fledged young ( $n = 38$ ). Therefore, yolk carotenoid concentrations may play an important role in Yellow-headed Blackbird reproductive success, but more detailed reproductive data and larger sample sizes ( $n > 100$ ) may be required to detect it.

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