

CAROTENOIDS IN THE EGGS OF AMERICAN COOTS: ASSOCIATIONS WITH
SIZE OF EGGS, LOCAL ENVIRONMENT, AND DIET

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ABSTRACT

I studied carotenoids in the eggs of American coots (*Fulica americana*) from 3 study sites in Saskatchewan, Canada. I supplemented two diet types designed to reduce carotenoids in the diet of laying coots to investigate the relationship of carotenoids and the size of eggs and to examine the allocation of carotenoids into eggs.

In chapter 2, I examined influences of local environment, food quantity and food quality on egg size. Carotenoid content and stable nitrogen and carbon isotopes in yolk were measured and used to elucidate whether variation in type of food eaten contributes to egg size. By analyzing isotopes in coot tissues, I confirmed that coots use endogenous lipid reserves for egg formation but not endogenous protein reserves, and the size of eggs is more dependent on exogenous sources of nutrients. My data demonstrate that carotenoids are not causal in egg size, but are components of natural, high quality diets.

Carotenoids are obtained through the diet and deposited into egg yolk. It has been hypothesized that concentrations and percentages of individual carotenoids can be labile and dependent on diets or maintained in an optimal balance to meet requirements of embryos. In chapter 3, I investigate deposition of carotenoids in egg yolk among nesting locales, among hens within a site and among treatments in a diet manipulation experiment. My data show maintenance in the percent composition of a suite of 3 important carotenoids, lutein, zeaxanthin and β -carotene, independent of scale of investigation and in contrast to other individual carotenoids that appear to vary in proportions based on diet. These results suggest that birds can maintain nutritional balances in their eggs despite variation in diets.

In chapter 4, I tested 3 hypotheses regarding the apportionment of carotenoids into egg yolk over the laying sequence. Without exception, concentrations of these nutrients have previously been shown to decline with egg sequence. In contrast to these findings, coots actually increased the carotenoid concentration in yolks over the laying sequence. My experimental evidence supports the explanation that this pattern of deposition depends on carotenoid availability to the laying female.

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Introduction

1.1.1 Carotenoid Chemistry

Over 600 carotenoids have been identified in nature (Bendich and Olson 1989). The presence of a hydroxyl group on two cyclohexene rings connected by a chain of hydrocarbons (the chromophore) provides a structure that can absorb both free radicals (defined as a species with an atom containing an unpaired valence electron (Oxtoby et al. 1994)) and visible light (Stradi 1998). Hence, carotenoids serve in photo-protective and energy-gathering functions in plants and photosynthetic micro-organisms (Britton 1995, Ursi et al. 2003, Telfer et al. 2003) by transferring excitation energy (Frank and Cogdell 1996) and quenching singlet oxygen and triplet chlorophyll (Young and Frank 1996). Animals ingest a broad spectrum of these lipid soluble nutrients and use them for a wide variety of biological functions. For example, roles include pro-vitamin A activity (Bendich and Olson 1989, Surai 2002), antioxidant activity (Surai et al. 2001c), anti-carcinogenic activity (Gerster 1993), vitamin E recycling (Surai, Peter F. 2002), and as pigments for colour in fish (Bourne et al. 2003, Grether et al. 2004), reptiles (Macedonia et al. 2000) and in birds (Brush 1990, Prum and Brush 2002, Navara and Hill 2003, Pryke and Andersson 2003).

1.1.2 Carotenoids, Colour and Birds

Carotenoid-based coloration is frequently used during visual displays to convey information by birds. For example, colour may be used in mate choice as an honest

signal of quality in adult birds (Hamilton 1982, Mateos and Carranza 1997, McGraw and Hill 2000). Colour may also signal quality of offspring to parents. Saino et al. 2000 demonstrated that parent Barn Swallows (*Hirundo rustica*) favoured nestlings with brighter carotenoid-based mouth coloration. The authors used an immune challenge to demonstrate that parents were providing preferential treatment to offspring that were able to maintain gape coloration without compromising immune function (for a similar study see Gotmark and Ahlstrom 1997). Lyon et al. (1994) reported parents of American Coots (*Fulica americana*) prefer to feed chicks that were more brightly coloured, and this parental choice resulted in higher growth rates and increased survival. Though most carotenoid-based coloration in birds is correlated with an individual's diet (e.g., Surai et al. 2001b), colour of chicks at hatching is contingent only on the pigment from within the egg.

1.1.3 Carotenoids and Eggs

The presence of carotenoids in the yolk of eggs has been known for nearly a century (Palmer and Kempster 1919); however, only recently have functional explanations for their existence in yolk been explored (Sutherland 1984, Surai et al. 2001a, Surai et al. 2001c). It has been suggested that at low oxygen tensions, similar to those in the egg, carotenoids act as antioxidants by quenching free radicals and protecting against lipid peroxidation (Surai et al. 2001c, Blount et al. 2002b). Indeed, a minimum quantity of carotenoids is necessary in the egg because extreme lipid peroxidation causes detrimental conditions including nutritional encephalomalacia (Budowski et al. 1987). Furthermore, it has been hypothesized that at levels of lipid peroxidation that do not cause encephalomalacia, behaviour and immune function could still be affected (Surai et al. 1999, Blount et al. 2000). Recent literature suggests that

greater inputs of carotenoids into the eggs results in correlated measurable benefits to chicks (e.g., immune function, Saino et al. 2003).

Understanding physiological and environmental limits of carotenoid deposition into eggs is important if fecundity is affected. Many factors can limit the maternal absorption of carotenoids and subsequently deposition into eggs. For example, carotenoids may be limited in the maternal diet (Bortolotti et al. 2003). Alternatively, their deposition may be under direct control due to an adaptive value (Blount et al. 2002a, Royle et al. 2003) or under indirect control through some aspect of physiology such as an interaction with hormones (Reed and Vleck 2001, Verboven et al. 2003). Limitations to the female's ability to access carotenoids (e.g., diet) could cause trade offs between needs for her own maintenance (e.g., antioxidant protection, immune function, and integument coloration) and the deposition into the egg yolk (Surai et al. 2001b).

Species-specific patterns of carotenoid deposition in egg yolks may exist. To date, few studies have examined inter- and intra-clutch variation of carotenoids in eggs. Life history traits (e.g., precocial versus altricial offspring) may dictate different strategies for the investment of carotenoids. Alternatively, there may also be differential allocation of carotenoids into eggs from stores and diet from birds of different species. For example, if a female gradually depletes carotenoid stores while depositing pigments into eggs, one may see a reduction in carotenoid composition over the laying sequence. The carotenoid concentration in yolks of Red-legged Partridges (*Alectoris rufa*) (Bortolotti et al. 2003), Lesser Black-backed Gulls (*Larus fuscus*) (Blount et al. 2000), Barn Swallows (*Hirundo rustica*) (Bernardo 1996), Zebra Finches (*Taenipygia guttata*)

(Royle et al. 2003), Great Tits (*Parus major*) (Hörak et al. 2002), a group of eight passerines (Cassey et al. 2005) and domestic chickens (*Gallus domesticus*) (Bortolotti et al. unpublished data) has been shown to decline over the laying sequence.

1.2 General Objectives

1.2.1 Influence of Diet Quality on Egg Size

Proximate explanations including environmental effects and differences in maternal diet (Winkler 1985) have been postulated for variation in the size of eggs. Several species of rail have been used in the past as model species to examine whether size of eggs is depends on food quantity, food quality, or both. However, results have been inconsistent. In chapter 2, I use food supplementation with analyses of carotenoids and stable isotopes in yolks to establish whether endogenous or exogenous sources of nutrients are used for egg formation, and whether there is a greater effect of diet quantity or quality on the size of eggs.

1.2.2 Dietary Effects on Proportions of Carotenoids in Eggs

In birds, carotenoids are not synthesized *de novo* but rather are obtained in the diet and subsequently deposited into egg yolks (Surai et al. 2001b, Bortolotti et al. 2003). To what degree the profile of individual carotenoids in the yolk are dependent on the composition of the diet (Carotenoid-Availability hypothesis), or whether they are regulated specifically by the hen (Recipe for a Good Egg hypothesis), is not well known. The objective of chapter 3 is to discriminate between these two hypotheses by examining both concentrations and percentages of various carotenoids in egg yolk using three scales of variation – among nests on different waterbodies, among hens within a locale, and among treatments in a diet manipulation experiment.

1.2.3 Why Do Yolk Carotenoid Concentrations Vary with Laying Sequence?

There are different strategies of allocating macro-nutrients to the egg over the laying sequence (Arnold et al. 1991, Nager et al. 2000); similarly, there may be different patterns of deposition of carotenoids in yolk over the laying sequence. Although few studies have been conducted to date, a pattern is emerging with regard to how females may be apportioning carotenoids among successive eggs in their clutch. Without exception, concentrations of these nutrients decline with egg sequence.

Two hypotheses have been suggested as explanations for the change in concentrations over the laying sequence. The Adaptive Maternal Effect hypothesis suggests that females differentially apportion carotenoids in favour of more valuable eggs. The Carotenoid Availability hypothesis considers that the concentration in eggs is a direct consequence of the amount of carotenoids in the diet and within a female's stores. I also propose the Physiological Artefact hypothesis, whereby differences in the concentrations of eggs are resultant of some aspect of correlative physiology. The objective of chapter 4 is to test these three hypotheses.

1.3 General Methods

1.3.1 Study Species

The American Coot is an ideal model for understanding the ecophysiology of antioxidants in eggs as certain attributes of their life history could necessitate the presence of carotenoids in eggs beyond those of previously studied species. Coots have relatively short prenatal growth periods, so have swift development (Gullion 1954). Increased metabolism associated with rapid growth may demand the protective role of carotenoids. Adult coots discriminate against chicks in their clutch by preferentially

feeding more brightly coloured offspring (Lyon et al. 1994). The source of pigments for this colour are carotenoids in the egg. Coots lay large clutches of 8-12 eggs (Arnold et al. 1991, Brisbin Jr. et al. 2002), but typically raise fewer than 4 chicks (Lyon 1993); therefore, the relative importance of carotenoids in the yolk for plumage colouration may be increased due to sibling competition.

American Coots are abundant, conspicuous game birds, showing their highest population densities in Saskatchewan (Alisauskas and Arnold 1994). Therefore, the large population base can readily absorb sampling without any negative effects and the logistics of sampling and experimentation are relatively convenient as the reproductive biology of the coot has been well studied. Coots build nests in emergent vegetation zones in wetlands of varying sizes (Gullion 1954, Sutherland and Maher 1987, Hill 1988, Brisbin Jr. et al. 2002). Nests are easy to locate (pers. obs.) and allow for easy egg collection (pers. obs., Alisauskas 1986). Supplemental feeding has been shown to affect eggs (Hill 1988, Arnold 1994), and there are baseline data on both coot egg composition (Alisauskas 1986, Arnold et al. 1991) and production (Arnold 1994). The behaviour of coots enables us to be certain where they have fed (Brisbin Jr. et al. 2002). Coots feed only within their territories (Gullion 1954), readily accept supplemental feed (Hill 1988, Arnold 1994) and rigorously defend territories against conspecifics (Gullion 1954).

1.3.2 Study Areas

Most work was conducted at two sites (Salt and Indi Lakes) in 2001 and at one site (Eyebrow Lake) in 2002, in south-central Saskatchewan, Canada. Salt Lake (51° 42' N, 70° 31' W) is a relatively small, 12-ha, 1 m deep, marsh that held water only

through July. Indi Lake (51° 41' N, 106° 31' W) is a large, 293-ha, open-water marsh (Messier et al. 1990). Indi Lake's water depth was maintained to at least 0.85 m by diverting water from an irrigation canal system. Eyebrow Lake (50°57' N 106°10' W) is a large, 875-ha, wetland along the floodplain of the Qu'Appelle River, with average water levels maintained at a depth of 1.5 m by a system of dykes. Proportions of vegetation varied between the sites, but prairie bulrush (*Scirpus spp.*), broadleaf cattail (*Typha latifolia*), and whitetop (*Scolochloa festucacea*) were generally present (Messier et al. 1990, Virgl and Messier 1992). I also incorporate data on eggs collected in 2000 at various wetlands in south-central Saskatchewan (Bortolotti unpubl. data).

1.3.3 Supplemental Feeding and Study Design

My experiment was designed to compare the effects of three food sources on the eggs of coots. The first source was the natural diet ("control diet"). Coots have among the highest carotenoid levels in their yolks of any bird (Surai et al. 2001d, Blount 2004). Therefore, unlike other food supplementation studies where carotenoid concentrations are enhanced, I attempted to reduce the availability of carotenoids. Thus, experimental food sources had a foundation of a grain-based duck breeder diet (Table 1.1) which is naturally low in carotenoids. They were steam-pelleted (University of Saskatchewan Feed Mill, Saskatoon, Saskatchewan) and formulated to 18.0% crude protein, 4.9% crude fat, 2.9% vitamins, and 1.0% phosphate resulting in 2.85 kcal/g of metabolically available energy. The "low carotenoid diet" was unaltered, but the "moderate carotenoid diet" contained a lutein supplement since it is the major carotenoid found in coot eggs (Surai et al. 2001d). The moderate carotenoid diet contained Flora Glo[®] Lutein beadlets (5 % lutein, 0.25 % Zeaxanthin, Kemin Foods L.C., Iowa, USA) at a

Table 1-1. Major constituents of duck breeder diet.

<u>Ingredient</u>	<u>Percentage</u>
Wheat	55.1
Soya Bean Mill	14.8
Barley	10.0
Canola Meal	8.0
Limestone	7.7
Tallow	3.4
Ca ₂ PO ₄	1.1
Vitamin/Mineral Pack	0.5
NaCl	0.23
Amino Acids	0.2

concentration of 50 mg lutein/kg pellets. Carotenoid dose was chosen after reviewing literature on poultry feeding experiments and calculating the ratio of maternal dietary carotenoids to egg carotenoids (Bortolotti et al. 2003) and targeting a concentration in the feed that resulted in yolks containing concentrations of carotenoids between those in the low carotenoid diet and control. A total carotenoid concentration of 1.66 and 16.55 $\mu\text{g/g}$ was measured (see below) from 1 sample of the low carotenoid and moderate carotenoid diets respectively.

The three wetlands were delineated in a randomized block design (Hicks 1982). Blocks contained three randomly allocated treatments (i.e., diet types) separated by a 50 m buffer zone to ensure birds only fed on their respective dietary treatment. Salt Lake contained two blocks, and Indi and Eyebrow lakes each had five blocks. Supplemental food was provisioned at Salt Lake as laying commenced, at Indi Lake immediately prior to clutch initiation, and at Eyebrow Lake approximately two weeks prior to clutch initiation. Food was replenished daily and placed on 60 cm X 60 cm, anchored, wooden board and Styrofoam floating platforms. Given that the range of territory size in coots is 0.03 ha to 0.56 ha (Gullion 1953, Sutherland 1984), we dispersed platforms at a density of 9 platforms/ha to ensure all coot pairs within the low and moderate carotenoid treatments had access to a supplemental food source within their territory.

1.3.4 Carotenoid Extraction and Analysis

Eggs from which carotenoids were to be extracted were frozen at -20°C . Frozen eggs were opened, and by using differential thawing of albumen and yolk, albumen was stripped and yolks weighed (Bortolotti et al. 2003). Frozen yolks were shipped on dry ice to the Avian Science Research Centre, Ayr, Scotland, where I extracted carotenoids (following Surai and Speake, 1998). Briefly, a sample of yolk or feed pellets

(approximately 0.4 g) was agitated in 0.7 ml of 5% NaCl solution for 30 s until yolk was in solution form. This solution was then homogenised for 2 min. One ml of methanol was homogenised into the solution to precipitate lipids. Two ml of hexane was added and homogenisation continued for 3 min. The solution was centrifuged and hexane (containing carotenoids) was collected. Hexane was added again for a second extraction which was combined with the first and evaporated under N₂. The residue was dissolved in 1 ml of methanol:dichloromethane (1:1 v/v), centrifuged and the supernatant was used for HPLC analysis. Using the same method, we extracted carotenoids from 1 sample of each diet type.

Carotenoids were determined using high performance liquid chromatography (HPLC) as previously described by Surai and Speake 1998. Dissolved carotenoids were injected into a Spherisorb type S3ODS2, 5- μ C18, reverse-phase column, 25 cm x 4.6 mm (Phase Separation, Clwyd, UK) with a mobile phase of acetonitrile-methanol (85:15) and acetonitrile-dichloromethane-methanol (70:20:10) in a gradient elution. Absorbance was detected at 445 nm. Peaks were identified by retention time comparison with known standards (variously obtained from Sigma, Poole, UK; Fluka, Gillinham, UK; Apin, Abingdon, UK; and Hoffman-La Roche, Basel, Switzerland). There were 13 regularly occurring peaks on the chromatograms that represented unidentifiable carotenoids. All were at low concentrations and the sum of their concentrations was used as a variable I refer to as a sum of unknowns.

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CHAPTER 2
INFLUENCE OF DIET ON EGG SIZE IN AMERICAN COOTS: EVIDENCE FROM
FOOD SUPPLEMENTATION AND BIOCHEMICAL MARKERS

2.1 Introduction

In a general review of birds, Christians (2002) pointed out that intraspecific variation in egg size can be attributed to differences among females' characteristics. These differences include body size, age and experience and can have slight positive influences on egg size in many species, as do some measures of female quality, as ascertained by measures of nest initiation date or clutch size (Massaro et al. 2002, Christians 2002). Measurements of the physiological state of females, including blood haemoglobin and hematocrit (Dufva 1996), estradiol (Christians and Williams 1999), follicle-stimulating hormone (Christians and Williams 2000) and body protein (Murphy 1986) have also been recognized as important covariates in predicting egg size (Christians 2002).

Within-female variation generally accounts for 30% of total egg size variation (Christians 2002), but in some species, it may be as high as 60% (Williams 1990). Of particular interest has been the evolution of adaptations related to laying sequence and egg size (Slagsvold et al. 1984, Arnold 1991, Kristensen et al. 2003). Two hypotheses for adaptive patterns of nutrient allocation to eggs are commonly evoked. The "brood reduction" strategy suggests that for some species the last-laid egg is smaller to reduce investment because of, or even to facilitate, the high probability of mortality of offspring from that egg (Forbes and Mock 2000). In contrast, the "brood survival" strategy

involves the laying of a larger last-laid egg to increase the probability of fledging success of young in that otherwise disadvantaged terminal position (Clark and Wilson 1981, Slagsvold et al. 1984, Hillström 1999).

While there are adaptive scenarios for sequence effects on egg size, there have also been proximate explanations postulated that include environmental effects and differences in maternal diet (Winkler 1985, Bolton et al. 1992, Reynolds et al. 2003a, Hargitai et al. 2005). Variation in maternal diets can be caused by differences in local environments between years at the same site and/or changes in food sources within the season (e.g., Murkin et al. 1997, Hargitai et al. 2005). If food availability and diet quality are altered, trade-offs in the use of energy and nutrients between those required by the female for her own use and what is required for reproduction can occur (Martin 1995, Vézina et al. 2003, Bureš and Weidinger 2003). These effects may then be manifested in both inter- and intra-clutch variation in egg size (Salvante and Williams 2002, Vézina et al. 2003). A major focus of studies investigating the effects of diet variation on reproduction has been the energy required for egg production (Jonsson 1997). As a result, researchers have manipulated maternal diets using either food supplementation (e.g., Arcese and Smith 1988, Bolton et al. 1992, Arnold 1992b, Wiebe and Bortolotti 1995, Reynolds et al. 2003b) or food reduction (Newton 1980, Rodenhouse and Holmes 1992). Egg size responses to manipulated diets have been variable (Magrath 1992); in some cases egg size increases with food availability (e.g., Wiebe and Bortolotti 1995), whereas in other cases it did not (Arnold 1992b, Nager et al. 1997). Results of diet manipulation experiments may be influenced less by food

availability than by diet quality, with the possibility that a specific dietary component drives egg size (Eldridge and Krapu 1988, Bolton et al. 1993, Rutstein et al. 2004).

The American Coot (hereafter coot) and its close relative the Eurasian Coot (*F. atra*), have proven to be model species for food supplementation experiments (Brinkhof et al. 1993). Coots feed within their territories (Gullion 1953, Reed and Vleck 2001) and readily accept supplemental feed (Hill 1988, Arnold 1994) which increases confidence that experimental birds will eat food provisioned within their territory. Coot nests can also be readily located allowing easy egg collection (Alisauskas 1986, Reed and Vleck 2001).

The response of coots to supplemental food experiments has been inconsistent. For example, Horsfall (1984) observed a reduction in variability in egg size for Eurasian Coots when fed grain- and corn-based diets. Hill (1988) demonstrated a positive egg size response when American Coots were provisioned with dog chow; however, Arnold (1994) failed to detect a response in egg size using a suite of diet types (trout chow, rabbit chow, fowl layer diet, and steam-rolled corn). Variation in laying date of coots due to diet manipulations similarly shows no consistent pattern among studies (see Horsfall 1984, Hill 1988, Arnold et al. 1991, Arnold 1994).

While food supplementation experiments focus on the manipulation of energy and macro-nutrients, only recently has a field experiment focused on manipulating micronutrients in the diet (Blount et al. 2002). Yolk carotenoids are deposited during egg formation (Palmer and Kempster 1919, Blount et al. 2000) and are necessary for the health of birds (Møller et al. 2000, Surai 2002) and for embryo viability (Budowski et al. 1987). Furthermore, carotenoids are not synthesised *de novo* and are only available

through the diet (Bendich and Olson 1989). Therefore, yolk carotenoid concentration should be an indicator of diet quality. It has been speculated that a lack of carotenoids may limit egg production (Royle et al. 1999, Blount et al. 2002, Blount et al. 2004) and supplementing carotenoids has been found to correlate with an ability of the female to re-lay (Blount et al. 2004). It is possible then, that a relationship between carotenoids in the diet and egg parameters exists (for potential mechanisms see Blount et al. 2004). For example, several studies using food supplementation have shown laying females with a diet of eggs or egg yolk lay larger eggs (Bolton et al. 1992, Ramsay and Houston 1997, Rutstein et al. 2004). Egg yolk has greater levels of dietary carotenoids than purely fat-based or seed-based supplements (Blount et al. 2002, Chung et al. 2004), hence low levels of carotenoids in the diet could be limiting the size of eggs.

Coots inhabit freshwater marshes that vary in the availability of aquatic plants and where aquatic insect emergences are unpredictable (Orians 1961, Verner and Willson 1966, Verner and Engelsen 1970, Ryan and Dinsmore 1979). Coot reproductive success depends on the spatial distribution of food (Ryan and Dinsmore 1979) and food quantity is important for chick survival (Lyon 1993). Thus, reproductive success, mediated by egg size, can differ among habitats of different quality. Hill (1988) found differences in size of coot eggs from different wetlands, and Reed (2000) observed that larger coot chicks from larger eggs had greater survival in their first 2 weeks compared to smaller chicks from smaller eggs.

I wanted to test whether egg size influences the fitness of female coots. I incubated coot eggs to identify whether larger chicks hatched from larger eggs. To determine the relative influence of environment versus food quantity and quality on coot egg size, I

conducted experiments on several wetlands. The sites were chosen to have different physiognomy and distinct differences in vegetative characteristics creating potential differences in habitat quality influencing clutch formation. Study sites are considered replicates to identify whether there were commonalities in the patterns of variables that had significant effects on the size of eggs. I tested the effects of diet quantity by provisioning birds with food. I examined the effects of food quality by manipulating dietary carotenoids and by measuring carotenoid concentrations in egg yolks. I further explored whether variation in the type of food eaten contributed to egg size by analysing stable isotopes of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) in yolks (Hobson 1995). Finally, I examined isotopically whether egg formation was driven from contributions of endogenous or exogenous resources using tissue and egg samples of adult coots (Hobson et al. 2000).

2.2 Methods

For general methods, see section 1.3.

2.2.1 Supplemental Feeding

My study was designed primarily to examine the consequences of carotenoids in eggs of American coots by means of an experiment comparing the effects of three food sources. The first source was the natural diet of coots (or “control diet”). Because coots have among the highest carotenoid levels in their yolks of any bird (Surai et al. 2001), the second and third sources were grain-based duck breeder diets designed to lower dietary carotenoids. I reasoned that eggs from the supplemented diets should be smaller if egg size is carotenoid dependent (Blount et al. 2004), but larger if quantity of food is important.

2.2.2 Egg Collection

Nests were located during early construction and monitored daily to identify time of clutch initiation. Once an egg was laid, it was labeled according to laying order, and since coots lay one egg per day (e.g., Lyon 1993, Arnold 1994), the nest was visited daily to verify sequence. Egg collection commenced when three eggs were laid in a nest, with the oldest egg collected daily. Conspecific dump eggs were identified by timing of laying and then by comparing appearance (as per Lyon 1993), and were excluded from these analyses. Maximum length and breadth were measured on all eggs with a digital calliper to estimate egg volume (Hoyt 1979).

Eggs collected during a preliminary study at Eyebrow Lake, Saskatchewan in 2000 were used for stable isotope analysis (see below), as were eggs from Salt and Indi lakes in 2001. Sampling protocol was different in the preliminary study in 2000 as sequence was not recorded; however, eggs were collected before clutch completion and so, on average, the egg was from sequence 4. For consistency, only the third or fourth egg was used for isotope analysis of eggs from Salt and Indi lakes.

2.2.3 Incubation Protocol

Three Marsh Roll-X incubators were used to incubate a total of 392 eggs. Eggs from each treatment were randomly placed in each incubator. Temperature in all three units was maintained at 36°C and humidity was kept at 80% and verified four times per day using a digital thermometer and a wet bulb thermometer, respectively. Every 24 h, eggs were lightly misted with water. An automatic turner rotated eggs every 4 h. Chicks were weighed within 8 h of hatch and dispatched by cervical dislocation. Measurements of skeletal morphology were taken (toe length, tarsus length, bill length, antebrachium length, wing chord length, and gape) to the nearest 0.1 mm, and were

incorporated into a principal component analysis to create a variable (PC2) representative of overall body size.

2.2.4 Stable Isotope Analysis

As part of a separate study conducted by Dr. Keith A. Hobson in 1995, 27 adult breeding coots were collected during the egg-laying period from 13 wetlands in the region. Muscle, liver, and abdominal fat were taken from these birds for carbon and nitrogen isotope analyses. Eggs and developing egg follicles from a subsample of these birds were taken for the analysis of egg components. The purpose of that collection was to investigate isotopic evidence for endogenous and exogenous nutrient inputs to eggs as per the approach of Hobson (1995) and Hobson et al. (2000). Simply, the measured egg isotope values were examined against predicted isotope values for each source. Tissues were kept frozen and prepared for stable isotope analysis as outlined in Hobson et al. (2000). To supplement these samples, I collected four additional birds from Eyebrow Lake in 2002 to examine stomach contents and run isotope analyses on those.

Lipids were extracted from yolk and abdominal fat samples using 2:1 chloroform: methanol solvent rinse and then freeze dried. Stable-carbon and nitrogen isotope assays were performed on homogenised materials by combusting 1 mg sub-samples at 1,800°C in a Europa ANCA-GST elemental analyser (Europa Scientific, Crewe, UK). Resultant CO₂ and N₂ gases were subsequently analysed using an interfaced Europa 20:20 continuous-flow isotope ratio mass spectrometer (Europa Scientific, Crewe, UK) with unknowns separated by laboratory standards (albumen). Stable isotope abundance was computed in δ -notation relative to carbonate Pee Dee Belemnite (carbon) and atmospheric air (nitrogen):

$$\delta^{15}\text{N or } \delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}})-1] \times 1000$$

where $R = (^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N})$ of the sample and standards, respectively. Analytical precision (± 1 SD) of these measurements is estimated to be $\pm 0.1\%$ and $\pm 0.3\%$ for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, respectively (Hobson et al. 1994, Hobson 1995).

2.2.5 Statistical Analyses

All effects on egg volume were evaluated using analysis of variance (ANOVA) in SAS (SAS Institute 1999). I assessed whether site influenced egg volume of the first-laid, control eggs. Block and nest were identified as random factors, and site was a fixed factor. My general statistical method for the remaining analyses examining the potential contribution of the variables of interest was to use analysis of covariance (PROC MIXED; SAS Institute 1999). Data were tested to confirm normality and then effects of relevant factors, covariates and 2-way interactions were modeled in all analyses. Beginning with interactions, non-significant effects were iteratively removed using a stepwise backwards procedure at a significance level set *a priori* at $P = 0.05$. I calculated degrees of freedom using the Satterthwaite method (SAS Institute 1999).

Marshes that are spatially separate and have observable variation in physical characteristics should also have a range in the quantity and types of food available (Gammonley and Laubhan 2002, Tangen et al. 2003); therefore, I assessed the local effects on egg volume on a per site basis. To test for the effect of diet on egg volume among clutches, the ANCOVA contained the random factors of block and nest to account for potential variation within the marsh and to control for pseudoreplication by sampling eggs within a nest (Fargallo et al. 2003). Treatment was considered as a fixed factor. Covariates included position in the laying sequence, total yolk carotenoids and

clutch initiation date. I also included a laying sequence * laying sequence term since preliminary plots showed a non-linear sequence pattern similar as reported by Arnold (1991). Clutch size was limited to eggs at or below laying sequence number 10 to exclude eggs from supernormal clutches potentially caused by feeding or removing eggs (Arnold et al. 1991, Arnold 1992a, Lyon 1993, Lyon 2003). A maximum clutch size of 10 eggs is within previously identified normal values (Hill 1988, Arnold 1992a, Lyon 2003).

2.3 Results

Bird and egg tissues from the 1995 collection of 27 adult coots from the region resulted in the following stable isotope values (mean \pm SD): muscle ($n = 27$, $\delta^{15}\text{N}$: $9.8 \pm 1.7\text{‰}$; $\delta^{13}\text{C}$: $-27.2 \pm 2.2\text{‰}$), abdominal fat ($n = 25$, $\delta^{13}\text{C}$: $-33.7 \pm 3.1\text{‰}$), egg lipids ($n = 15$, $\delta^{13}\text{C}$: $-32.5 \pm 2.3\text{‰}$), and egg yolk ($n = 21$, $\delta^{15}\text{N}$: $9.8 \pm 2.6\text{‰}$; $\delta^{13}\text{C}$: $-26.5 \pm 1.0\text{‰}$). The body tissue data allow the prediction of the egg tissue values to be expected from endogenous reserves according to the model described in Hobson (1995). For protein, I expected that lipid-free yolk should have a mean $\delta^{15}\text{N}$ value of 13.2‰ and a $\delta^{13}\text{C}$ value of -27.2‰ , if derived entirely from endogenous (muscle) protein reserves. For yolk lipids, I predicted $\delta^{13}\text{C}$ values to be the same as those in abdominal fat (i.e. no isotopic discrimination), namely around -33.7‰ , a value not significantly different from my mean egg lipid value of -32.5‰ ($t = 1.6$, $P = 0.2$). Because I did not conduct an exhaustive isotopic assay of the local foods available to or used by coots, isotopic endpoints expected for eggs derived entirely from exogenous sources in my study area could not be confidently derived. However, the isotopic measurement of dietary materials from collected coots averaged $5.6 \pm 1.5\text{‰}$ for $\delta^{15}\text{N}$ ($n = 8$) but varied

considerably for $\delta^{13}\text{C}$ values (fine seed and plant debris: $-25.2 \pm 1.8\text{‰}$, $n = 5$; *Myriophyllum* spp.: -18.5‰ , $n = 1$; *Utriculina* spp.: -30.5‰ , $n = 2$). For the $\delta^{15}\text{N}$ data, egg protein derived from these exogenous sources would show mean values of about 9 ‰, whereas $\delta^{13}\text{C}$ values could range from -30.5 to -18.5‰ , encompassing the values I measured for eggs.

Mean (\pm SE) egg volumes of first-laid, control eggs at Salt Lake ($28.0 \pm 1.73\text{ cm}^3$, $n = 10$), Indi Lake ($28.9 \pm 2.60\text{ cm}^3$, $n = 15$) and Eyebrow Lake 2002 ($27.0 \pm 2.61\text{ cm}^3$, $n = 55$) were similar to those previously reported for the American Coot (Arnold 1994). The volumes of first-laid, control eggs were not significantly different among sites ($F_{2,62.9} = 1.72$, $P = 0.188$). Overall, larger eggs produced chicks of greater structural size (ANCOVA, PCA chick size: $F_{1,131} = 25.8$, $P < 0.0001$).

There were unique combinations of significant variables influencing the volume of eggs within each site (Table 2-1). Since Eyebrow Lake was the only site where egg volume was significantly affected by treatment, I investigated whether the relationship between carotenoids and egg volume was causal or merely correlative. I limited the analysis to the two supplemented diets, and thereby controlled for quantity of food (i.e., coots had similar access to provisioned macronutrients). Total carotenoids ($F_{1,136} = 4.23$, $P < 0.042$) and laying sequence ($F_{1,107} = 4.63$, $P < 0.034$) continued to have significant effects on egg volume, but treatment was not significant (iteratively removed at $F_{1,5.84} = 2.93$, $P = 0.139$) suggesting a component in the local environment associated with carotenoids, but not carotenoids per se, was responsible for egg volume.

Egg isotope values are summarized in Table 2-2. Yolk $\delta^{15}\text{N}$ values did not significantly contribute to the model of egg volume ($\delta^{15}\text{N}$: $F_{1,30} = 0.00$, $P = 0.995$). Site

and yolk $\delta^{13}\text{C}$ value both had significant effects (site: $F_{2,31} = 5.634$, $P = 0.008$; $\delta^{13}\text{C}$: $F_{1,31} = 13.69$, $P = 0.001$) (Fig. 2-1). Moreover, carotenoids were associated with yolk $\delta^{13}\text{C}$ values (ANCOVA: $F_{1,23} = 7.5$, $P = 0.001$) suggesting that the same aspect of food quality that varies with $\delta^{13}\text{C}$ values may also be influencing carotenoids. Both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were negatively correlated with clutch initiation date at Indi Lake ($\delta^{13}\text{C}$, $r = -0.554$, $P = 0.011$; $\delta^{15}\text{N}$, $r = -0.456$, $P = 0.043$), but neither correlated with clutch initiation date at Salt Lake. Partial correlations between volume and clutch initiation date, controlling for $\delta^{13}\text{C}$ values, resulted in a non-significant association at Indi Lake ($r = 0.0668$, $P = .786$, $n = 17$), but the partial correlation between volume and $\delta^{13}\text{C}$ values remained significant after controlling for clutch initiation date ($r = 0.5157$, $P = 0.024$, $n = 17$).

Table 2-1: ANCOVA statistics for effects on egg volume in American Coots at three study sites in Saskatchewan

Variable	Salt Lake			Indi Lake			Eyebrow Lake		
	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>
Laying Sequence	–	–	–	3.73	1,269	0.055	7.26	1,171	0.0078
(Laying Sequence * Laying sequence)	19.80	1,138	<0.0001	23.29	1,248	0.0001	–	–	–
Clutch Initiation Date	–	–	–	13.58	1,108	0.0004	–	–	–
Total Carotenoids	–	–	–	–	–	–	0.99	1,195	0.3208
Treatment	–	–	–	–	–	–	5.04	2,182	0.0074
(Clutch Initiation Date * Laying Sequence)	–	–	–	6.42	1,270	0.012	–	–	–
(Laying Sequence * Total Carotenoids)	–	–	–	–	–	–	4.74	1,165	0.0309
(Treatment * Total Carotenoids)	–	–	–	–	–	–	6.15	2,202	0.0026
Random Factors	<i>Z</i>	Pr <i>Z</i> ($\alpha = 0.05$)		<i>Z</i>	Pr <i>Z</i> ($\alpha = 0.05$)		<i>Z</i>	Pr <i>Z</i> ($\alpha = 0.05$)	
Block	–	–		0.78	0.219		0.70	.242	
Nest	3.03	.001		4.56	<0.0001		4.62	<0.0001	

Table 2-2: Stable isotope values of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) (mean \pm SD and ranges) for eggs of American Coots at three study sites in Saskatchewan.

Site	N	$\delta^{13}\text{C}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{15}\text{N}$ (‰)
		Mean \pm SD	Range	Mean \pm SD	Range
Salt Lake	8	-28.17 \pm	-29.22 to	9.26 \pm	8.68 to 9.88
		0.83	-28.17	0.42	
Indi Lake	20	-25.44 \pm	-27.12 to	9.80 \pm	7.12 to
		1.18	-23.32	1.18	11.75
Eyebrow Lake	7	-28.27 \pm	-29.99 to	9.16 \pm	7.96 to
		1.03	-26.89	1.13	11.49

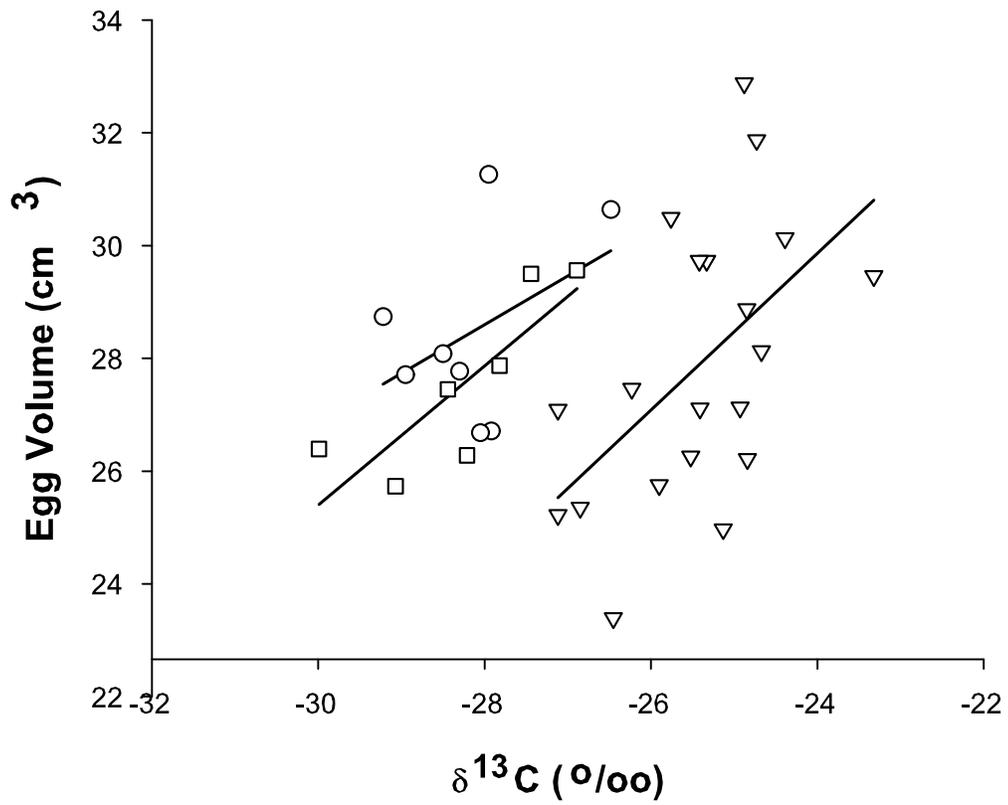


Figure 2-1: Scatterplot of $\delta^{13}\text{C}$ and Egg Volume in American Coots at three study sites. Squares represent Eyebrow Lake, 2000, triangles represent Indi Lake, 2001, and circles represent Salt Lake, 2001.

2.4 Discussion

Previous studies using conventional approaches to examine the relative inputs of endogenous and exogenous sources of nutrients in eggs of coots have suggested strong evidence for endogenous lipid inputs to the clutch, but relatively minor inputs of endogenous protein reserves (Alisauskas and Ankney 1985). My isotopic analysis supports the contention that endogenous lipid reserves are the source of lipids for eggs since the $\delta^{13}\text{C}$ values of abdominal fat closely resembled those of egg lipids, despite the potential for considerable variation in dietary $\delta^{13}\text{C}$ values. The $\delta^{15}\text{N}$ measurements are also consistent with the suggestion that coots derive protein for egg formation largely from exogenous sources since the measured mean egg protein $\delta^{15}\text{N}$ value of 9.8 ‰ corresponds closely with that predicted from my limited dietary samples. Future studies using stable isotopes to trace endogenous and exogenous nutrient inputs to coot eggs will face the challenge that freshwater aquatic habitats can have dietary samples with diverse isotopic signals. It is therefore difficult to determine precise isotopic endpoints in isotopic mixing models (e.g., Gauthier et al. 2003). For this study, the most parsimonious interpretation of the data is that coots mobilize little endogenous protein reserves for eggs, but may well rely on body lipid reserves for egg formation. It appears that local protein may be more limiting than dietary carbohydrates for egg size; however, physiological factors associated with birds arriving from their breeding ground, namely abdominal lipid reserves, may also potentially play a role.

The results of analyses on the size of eggs from this and previous studies support the scenario of deposition of the major components of an egg from separate sources. It has been suggested that coots must store a minimum of 27.5 g of abdominal fat to

initiate breeding (Alisauskas and Ankney 1985), this concept of a threshold is substantiated by my results demonstrating similarity in the size of first-laid eggs among study sites. American Coots nest in marsh habitats that vary in types of available food (e.g., Orians 1961, Hill 1988, Turner and McCarty 1998, Fletcher Jr. and Koford 2004, Gunnarsson et al. 2004), and as my results support that local sources of protein are deposited into eggs, then variation in available protein within respective waterbodies should be evident in the size of later-laid eggs. Indeed, Hill (1988) established that local food abundance at different sites influenced mean egg volume of a clutch, and Arnold (1991, 1994) found year, clutch initiation date, sequence, and food abundance were significant. My results (Table 2-1) also show substantial variation in the patterns of variables that affect the size of eggs. The sole consistent factor was an initial increase, then a decrease, in egg volume over the laying sequence (Arnold 1991, Reed and Vleck 2001). Furthermore, the location of the egg volume peak on average shifted from fourth in the laying sequence, to fifth or sixth in the supplemented birds which may be a response to factors associated with the adaptive value of hatching asynchrony in this species (Alisauskas 1986, Lyon 1993, Budden and Beissinger 2005). Collectively, the results from all studies suggest local differences in either the quantity of food or the quality of the diet available to breeding coots are influencing the size of eggs.

Both Arnold (1991, 1994) and Hill (1988) manipulated the quantity of food available to breeding coots but found that much of the variance in the size of eggs had yet to be explained. Likewise, I provisioned an *ad libitum* food supply that should have provided an excess of gross macronutrients, and I only observed a treatment effect at one of the three sites (i.e. Eyebrow Lake) (Table 2-1). However, the dual association of

both treatment and total carotenoids at Eyebrow Lake was indicative of an effect of diet quality. In the analysis of all eggs from Eyebrow Lake, total carotenoids was a significant covariate. However, the lack of effects on the size of eggs when carotenoids were experimentally added to the diet shows that carotenoids were not causal. Therefore, a more likely explanation for the treatment effect observed at Eyebrow Lake (Table 2-1), is that egg size was driven by a component in the natural diet that correlates with carotenoids, that is diet quality, rather than carotenoids *per se*.

Further support for a diet quality hypothesis came from the analysis of stable isotopes of carbon and nitrogen. There was a significant effect of lipid-free yolk $\delta^{13}\text{C}$ values on egg size (Fig. 2-1). Interestingly, the relationship between yolk protein $\delta^{13}\text{C}$ value and clutch initiation date paralleled those of clutch initiation date and volume within the sites. That is, clutch initiation date both correlated with yolk $\delta^{13}\text{C}$ values and had a significant effect on egg size at Indi Lake (Table 2-1). There was no correlation between clutch initiation date and yolk $\delta^{13}\text{C}$ values or any relationship between clutch initiation date and egg size at Salt Lake (Table 2-1). The subsequent partial correlation indicated that size of eggs was likely associated with a source of dietary carbon rather than clutch initiation date. Though a few experiments have focussed on diet quality (e.g., Bolton et al. 1993) and others on maternal quality (reviewed by Williams 1994), none has considered how changes in diet over the reproductive season may affect eggs, and subsequently offspring. These results open up a novel spectrum of costs to consider when examining questions regarding the optimal time for a female to nest.

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CHAPTER 3
ARE CAROTENOID PROFILES OF EGG YOLKS IN BIRDS REGULATED?

3.1 Introduction

Carotenoids are bioactive pigments that exist in an astonishing variety of molecular forms, with over 600 identified to date (Olson and Krinsky 1995). Carotenoids are synthesised by photosynthetic micro-organisms, algae, and plants (Olson and Krinsky 1995) and then ingested by animals for use in a wide variety of physiological processes (Goodwin 1986, Blount 2004). Of particular importance is the role of carotenoids as antioxidants that prevent lipid peroxidation, and reduce cytotoxicity and DNA breakage (reviewed by Surai 2002). In birds, carotenoids have been shown to be responsible for coloration (McGraw and Hill 2000, Faivre et al. 2003) and their antioxidant activity has been associated with attributes of health, especially immune function (e.g., Blount et al. 2003, Alonso-Alvarez et al. 2004). Within eggs, carotenoids are deposited in yolk lipids where they provide colour and fulfill a crucial role of providing antioxidant protection to vulnerable embryonic tissues (Blount et al. 2000, Surai et al. 2001a).

Individual carotenoids and their concentrations within eggs have been described for a very limited number of species (Speake et al. 1999), and empirical evidence suggests that birds may metabolically transform carotenoids of one type into another prior to deposition into yolk (Surai et al. 2001b, Blount et al. 2002a). It has been demonstrated in poultry that the transfer of each type of carotenoid to various embryonic tissues differs from relative amounts within the yolk (Surai et al. 2001b). In addition, *in vitro* studies have demonstrated different antioxidant capabilities of carotenoids

(reviewed by Surai 2002). Together, this evidence is suggestive of specialized requirements for specific carotenoids in different tissues (Surai et al. 2001a). Considering the competitive interactions between carotenoids (reviewed in Surai 2002) and potential for different requirements of each individual carotenoid in embryonic tissues (Surai et al. 2001b), the proportion of each carotenoid present in egg yolk may be as important as absolute concentration. However, despite the apparent importance of specific carotenoids, there is only limited information on variation in total carotenoids concentrations between populations (Hörak et al. 2002) and virtually no information on variability of concentrations and percentages of individual carotenoids within a species, beyond the variation among eggs within individual study populations (e.g., Saino et al. 2002, Blount et al. 2002b).

It is accepted that carotenoids are only available through the diet (Olson and Krinsky 1995) and that post-ingestion metabolic conversion is common (McGraw and Schuetz 2004). It has been postulated that there may be a prescription for an optimal balance of carotenoids to meet the requirements for embryonic development (Blount et al. 2002a). Several studies on species in captivity, especially poultry, have correlated carotenoids in the diet and in the egg (reviewed in Surai 2002, also see figure 1 in Bortolotti et al. 2003) and it has been hypothesised that these proportions of individual carotenoids in yolk are labile and depend on diet (Partali et al. 1987, Blount et al. 2002a). Alternatively, Blount (2004) suggested a “recipe for a good egg” hypothesis, where relative proportions of yolk carotenoids are maintained independently of diet by virtue of metabolic conversion or selective deposition by the female. Metabolic conversion by the female occurs prior to deposition into yolk in poultry (Goodwin

1986); furthermore, Lesser Black-backed Gulls (*Larus fuscus*) appear to partition individual carotenoids in predictable proportions despite variation in total carotenoid concentrations (Blount et al. 2002a). It follows then, that a cost of reproduction could be the maintenance of this equilibrium (Blount et al. 2002a).

The objective of this chapter is to discriminate between the two competing hypotheses regarding relative percentages of yolk carotenoids. My model species was the American coot (*Fulica americana*), an abundant water bird. Coots are opportunistic and generalist feeders, with a diet dominated by aquatic vegetation, but including aquatic vertebrates and invertebrates, and terrestrial vegetation (Brisbin Jr. et al. 2002). This dietary diversity should allow for considerable variation in ingested carotenoids. To differentiate between the hypotheses, I examined carotenoid profiles in eggs among sites (i.e. waterbodies) which should represent the broadest variation in diet. I then examined profiles among hens within a site, and among the treatments of a diet manipulation experiment designed to alter the availability of carotenoids. I predicted that if coots deposit carotenoids into their yolk in percentages depending on diet, then proportions should vary among and within sites and by treatment. Alternatively, if proportions of individual carotenoids are regulated, then the relative percentages should be consistent.

3.2 Methods

For general methods, see section 1.3.

3.2.1 Study Areas and Egg Collection

Eggs were collected in 2000 through 2002 from eight waterbodies in central Saskatchewan, Canada, within a geographic area roughly 200 km X 60 km (50° 30' N to

52° 43' N and 106° 00' W to 107° 26' W). The sites varied in size, structure and vegetation to encompass much of the extent of variation in natural nesting habitats of the coot. The waterbodies ranged from small (< 1-ha) ditches, to large (875-ha) wetlands, with vegetation as various combinations of great, common great, and prairie bulrush (*Scirpus spp.*), broadleaf cattail (*Typha latifolia*), whitetop (*Scolochloa festucacea*), and flooded hay fields. Furthermore, freshwater marshes are known to have erratic emergence of aquatic insects which can create variation in the diet of coots (e.g., Orians 1961; Verner & Engelsen 1970). Three of the sites were used in a more extensive study and so larger sample sizes were available and within those sites an experiment was executed to compare the effects of three food sources on the eggs of coots. Therefore, food was supplemented using the methods described in chapter 1. The sampling protocol for the three major sites differed from the others as nests were visited daily in order to identify the laying sequence of collected eggs; however, only one randomly selected egg per nest was used for analysis here. At the five minor sites, one egg was collected arbitrarily from nests with incomplete clutches (Hargitai *et al.* 2005) (i.e. less than 10 eggs, Arnold 1994).

3.2.2 Data Analysis

For comparison among sites, I limited my data set to one random egg per nest from the five minor sites and one random control egg per nest at the three major locales where the experiment was performed. I entered the concentrations of the six identifiable carotenoids and the sum of the unknown carotenoids from each egg into a principal component analysis (PCA) to reduce the dimensionality of the data set (Tabachnick & Fidell 1983). Due to sample size limitations, I tested for site differences using the

resultant, normally distributed PC scores from Salt Lake, Indi Lake and Eyebrow Lake, and each individual carotenoid, using analysis of variance (ANOVA). For all data, including one random egg from each nest in the treatment groups at Eyebrow Lake, I calculated the percent of the total of each individual carotenoid and the sum of the unknowns at each site, and used correlations to identify relationships between concentrations and relative proportions of individual carotenoids.

3.3 Results

Two factors explaining 56.7% and 20.4% of the variance were obtained from the PCA (Table 3-1). PC1 can be interpreted as a variable largely influenced by quantities of lutein, β -carotene, zeaxanthin, β -cryptoxanthin and the sum of unknowns, while PC2 can be explained by concentrations of canthaxanthin and echinenone (Table 3-1). For the three main wetlands, site was a significant factor in explaining the variation within both PC1 and PC2 (ANOVA PC1, site: $F_{2,51} = 5.8$, $p = 0.002$, ANOVA PC2, site: $F_{2,51} = 12.2$, $p < 0.001$) (Fig. 3-1), and when comparing the individual carotenoids, site again had a significant effect (Table 3-2).

I compared the relative variation of the means of the carotenoid concentrations and of the percentages of each carotenoid using coefficients of variation (CV_s) (Table 3-2). With the exception of canthaxanthin which was present only in small quantities, $CV_{concentration}$ ranged from 71.3 to 102.5 (Table 3-2). In contrast, the $CV_{percent}$ of the same samples were lower and ranged from 5.3 to 70.5 (Table 3-2). Three carotenoids in particular (lutein, zeaxanthin, and β -carotene), have a low $CV_{percent}$, both relative to their own concentrations and relative to canthaxanthin, β -cryptoxanthin, echinenone, and the sum of unknowns. Here, and in subsequent analyses, these two groups of carotenoids appear to be consistently different and I refer to the group containing lutein, zeaxanthin,

and β -carotene as low and the remaining carotenoids are collectively referred to as the high group.

To investigate variation among hens, I examined eggs from Eyebrow Lake, as it was my largest sample per wetland. The CV_{percent} was lower than the $CV_{\text{concentration}}$ for nearly all individual carotenoids. The low group of lutein, zeaxanthin, and β -carotene again had a low CV_{percent} compared to the remaining carotenoids (Table 3-3). However, there was little variation when comparing the $CV_{\text{concentration}}$ of all individual carotenoids (Table 3-3).

Table 3-1: Relative loadings of each factor from principal component analysis of the concentrations of individual carotenoids in the yolks of American coot eggs.

Variable	PC1	PC2
Lutein	0.900	-0.280
Zeaxanthin	0.926	-0.107
β -carotene	0.953	0.065
β -cryptoxanthin	0.734	0.313
Echinenone	0.124	0.798
Canthaxanthin	-0.076	0.776
Sum of Unknowns	0.913	0.022

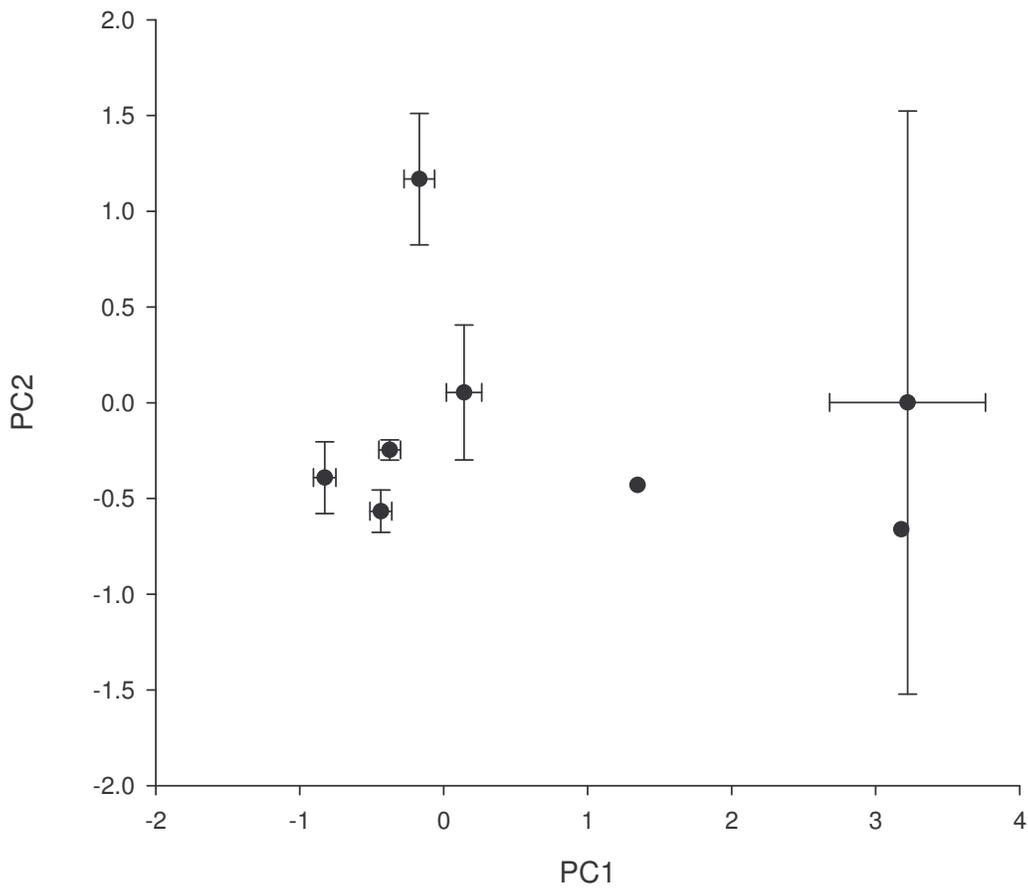


Figure 3-1. Mean \pm SE values for PC1 and PC2 for yolk concentration of carotenoids in the eggs of American coots from eight study sites in Saskatchewan. From left to right, study sites and sample sizes (n) are as follows: Kettlehut Lake ($n = 8$), Duck Lake ($n = 3$), Eyebrow Lake ($n = 31$), Salt Lake ($n = 11$), Indi Lake ($n = 13$), Montgomery Marsh ($n = 1$), Osler Marsh ($n = 1$), Rice Lake ($n = 3$).

Table 3-2. Carotenoid concentrations (mean $\mu\text{g/g} \pm \text{SE}$, %) in the yolks of eggs of American coots from eight study sites in Saskatchewan.

Site	Lutein		Zeaxanthin		β -carotene		Canthaxanthin		β -cryptoxanthin		Echinenone		Sum of unknowns	
	\bar{X} (SE)	%	\bar{X} (SE)	%	\bar{X} (SE)	%	\bar{X} (SE)	%	\bar{X} (SE)	%	\bar{X} (SE)	%	\bar{X} (SE)	%
Salt Lake (11)	6.41 ^a (0.44)	15.3	4.66 ^{ab} (0.66)	11.1	9.25 ^{ab} (1.39)	22.0	0.44 ^a (0.10)	<0.1	8.58 ^a (1.85)	20.4	2.18 ^a (0.33)	5.2	10.46 ^a (1.29)	24.9
Indi Lake (13)	16.20 ^b (2.43)	29.2	6.51 ^a (0.77)	11.7	12.82 ^a (1.34)	23.1	0.18 ^b (0.06)	<0.1	5.97 ^a (1.64)	10.8	1.53 ^a (0.56)	2.8	12.28 ^a (1.46)	22.1
Eyebrow Lake (31)	7.13 ^a (0.66)	22.3	3.90 ^b (0.37)	12.2	8.55 ^b (0.82)	26.7	0.04 ^b (0.00)	<0.1	6.10 ^{ba} (0.93)	19.1	1.25 ^a (0.12)	3.9	5.02 ^b (0.75)	15.7
Kettlehut Lake (8)	8.10 (0.59)	19.8	3.53 (0.22)	8.3	7.48 (0.92)	17.3	0.05 (0.01)	0.1	3.55 (1.13)	7.8	1.18 (0.54)	2.5	2.10 (0.33)	4.8
Duck Lake (3)	19.75 (1.48)	29.0	7.01 (0.43)	10.7	15.85 (0.99)	23.1	0.13 (0.02)	0.2	8.26 (1.01)	11.9	1.80 (0.33)	2.8	14.29 (0.89)	22.3
Highway Marsh (1)	17.77	17.6	15.12	15.0	21.98	21.8	ND	0	20.87	20.7	ND	0	25.29	25.0
Osler Marsh (1)	25.16	14.9	32.95	19.6	46.62	27.7	ND	0	33.40	19.8	ND	0	30.33	18.0
Rice Lake (3)	55.00 (5.11)	28.2	41.72 (1.21)	21.6	34.93 (2.67)	19.2	ND	0	15.46 (1.80)	8.3	4.30 (4.30)	3.0	35.49 (0.78)	19.6
Mean*	19.44	22.0	14.43	13.7	19.69	22.6	0.11	0.2	12.77	14.8	1.53	2.5	16.9	19.1
CV _{mean} *	81.6	27.6	102.5	33.2	72.0	5.3	1.43	1.76	79.0	38.2	89.2	70.5	71.3	34.7

ND = not detected, * Calculated among sites

Values of the first three sites with different superscripts are significantly different ($p < 0.05$) after Bonferroni correction with respect to row ($F_{2,53}$)

The concentration of total carotenoids differed among the treatment groups at Eyebrow Lake (ANOVA total carotenoids: $F_{2,97} = 3.3$, $p = 0.042$). Post-hoc Bonferroni adjusted comparisons indicated greatest differences between the control and low-carotenoid groups (Fig. 3-2). Just as I examined variation among sites and among hens, I compared dietary regimes at Eyebrow Lake (Table 3-4) using the $CV_{\text{concentration}}$ and the CV_{percent} (Table 3-5). Provisioning food increased the $CV_{\text{concentration}}$ for most of the individual carotenoids as would be expected since hens undoubtedly consumed both supplemented and at least some natural foods (Table 3-5). As before, $CV_{\text{concentration}}$ was high for all individual concentrations relative to the CV_{percent} regardless of treatment, but this was especially true for lutein, zeaxanthin and β -carotene. Lutein showed a remarkably similar CV_{percent} among all treatments even though this carotenoid was directly manipulated.

If there is a “recipe for a good egg” and the proportions of carotenoids are maintained despite variation in diet, then the percentage of each carotenoid should be independent of their concentrations. Alternatively, if proportions of carotenoids are labile and dependent on the diet, then there may be a relationship between the percentage and the respective concentrations of each individual carotenoid. My test used control eggs from Eyebrow Lake as it was my largest, single sample. There were significant correlations for canthaxanthin ($r_s = 0.667$, $p < 0.001$, $n = 31$), β -cryptoxanthin ($r_s = 0.883$, $p < 0.001$, $n = 31$) (Fig. 3-3), and echinenone ($r_s = 0.375$, $p = 0.038$, $n = 31$), which were all in the high variability group. β -carotene also positively

correlated ($r_s = 0.405$, $p = 0.024$, $n = 31$); however, the relationship was driven by two outliers which when removed weakened the correlation ($r_s = 0.274$, $p = 0.151$, $n = 29$).

Table 3-3. Coefficients of variation for seven individual carotenoid concentrations (mean $\mu\text{g/g} \pm \text{SE}$) in the yolk from control eggs of American coots from Eyebrow Lake, Saskatchewan ($n = 31$).

Carotenoid	CV _{concentration}	CV _{percent}
Lutein	49.3	16.3
Zeaxanthin	49.1	11.8
β -carotene	52.9	22.3
Echinenone	55.0	48.7
β -cryptoxanthin	82.2	48.1
Canthaxanthin	64.6	100.5
Sum of unknowns	73.9	44.2

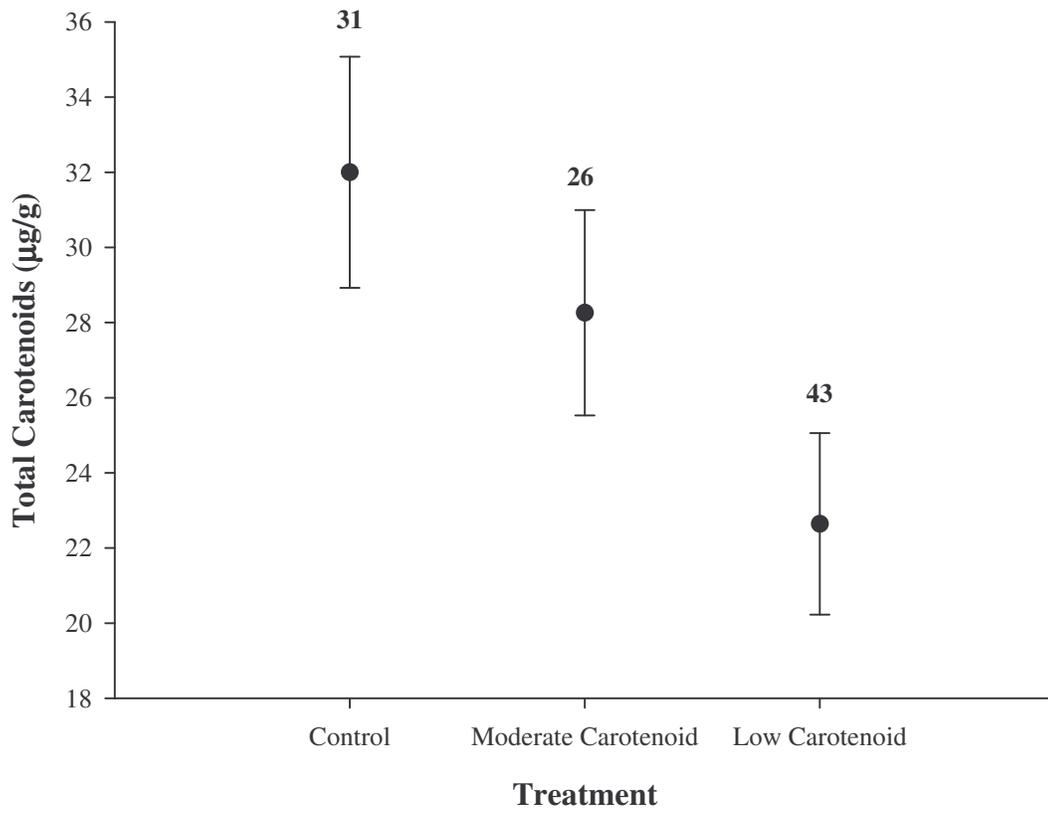


Figure 3-2. Mean \pm SE of total carotenoid concentrations in yolk of American coots from three treatments at Eyebrow Lake, Saskatchewan, Canada.

Table 3-4. Carotenoid concentrations (mean $\mu\text{g/g} \pm \text{SE}$, %) in the yolk of eggs of American coots on three diets at Eyebrow Lake, Saskatchewan, Canada.

Treatment	Lutein		Zeaxanthin		β -carotene		Canthaxanthin	β -cryptoxanthin		Echinenone		Sum of Unknowns		
	\bar{X} (\pm SE)	%	\bar{X} (\pm SE)	%	\bar{X} (\pm SE)	%		\bar{X} (\pm SE)	%	\bar{X} (\pm SE)	%	\bar{X} (\pm SE)	%	
Control (<i>n</i> = 31)	7.13 (0.66)	22.3	8.55 (0.82)	26.7	3.90 (0.37)	12.2	0.04 (0.00)	< 0.1	6.10 (0.93)	19.1	1.25 (0.12)	3.9	5.02 (0.75)	15.7
Moderate Carotenoid (<i>n</i> = 26)	6.94 (0.66)	24.3	7.38 (0.88)	25.9	3.39 (0.32)	11.9	0.05 (0.01)	< 0.1	4.96 (0.63)	17.4	1.18 (0.13)	4.1	4.65 (0.52)	16.3
Low Carotenoid (<i>n</i> = 43)	5.36 (0.60)	23.2	6.09 (0.75)	26.3	2.87 (0.31)	12.4	0.06 (0.01)	< 0.1	3.48 (0.47)	15.0	1.13 (0.20)	4.9	3.90 (0.36)	16.8

Table 3-5. Coefficients of variation for seven individual carotenoid concentrations and percents in the yolk of eggs of American coots on three diets at Eyebrow Lake, Saskatchewan.

Carotenoid	$CV_{\text{concentration}}$			CV_{percent}		
	Control	Moderate	Low	Control	Moderate	Low
Lutein	49.3	48.2	73.7	16.3	17.1	16.7
Zeaxanthin	49.1	47.7	71.1	11.8	9.7	16.1
β -carotene	52.9	60.5	80.7	22.3	24.9	30.8
Echinenone	55.0	57.4	114.4	48.7	64.3	74.1
β -cryptoxanthin	82.2	65.2	87.7	48.1	38.3	40.2
Canthaxanthin	64.6	61.0	77.2	100.5	85.9	142.1
Sum of Unknowns	73.9	57.2	59.9	44.2	29.3	40.7

The percentages of lutein ($r_s = 0.103$, $p = 0.328$, $n = 31$) (Fig. 3-3), zeaxanthin ($r_s = -0.76$, $p = 0.685$, $n = 31$) and sum of unknowns ($r_s = 0.078$, $p = 0.676$, $n = 31$) were not correlated with concentrations.

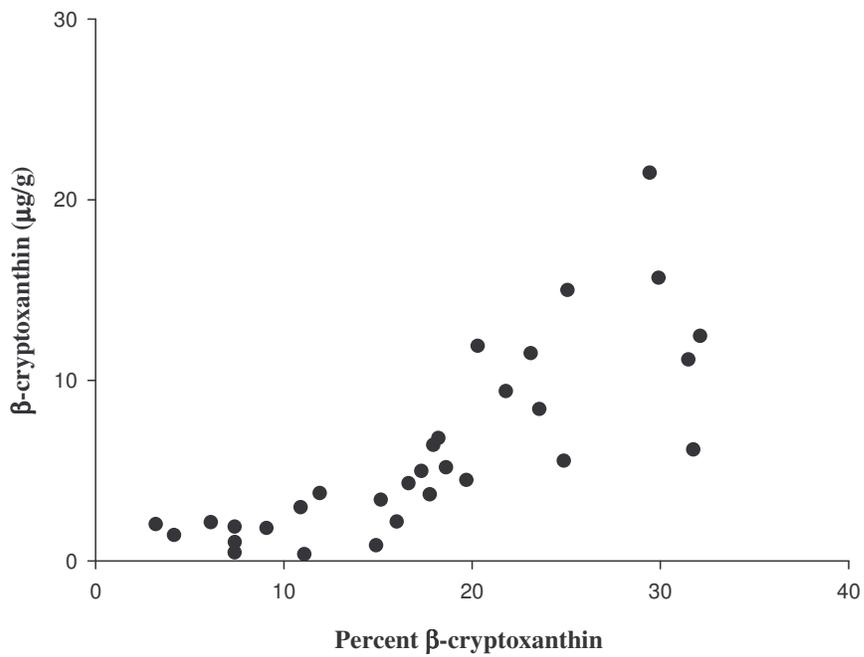
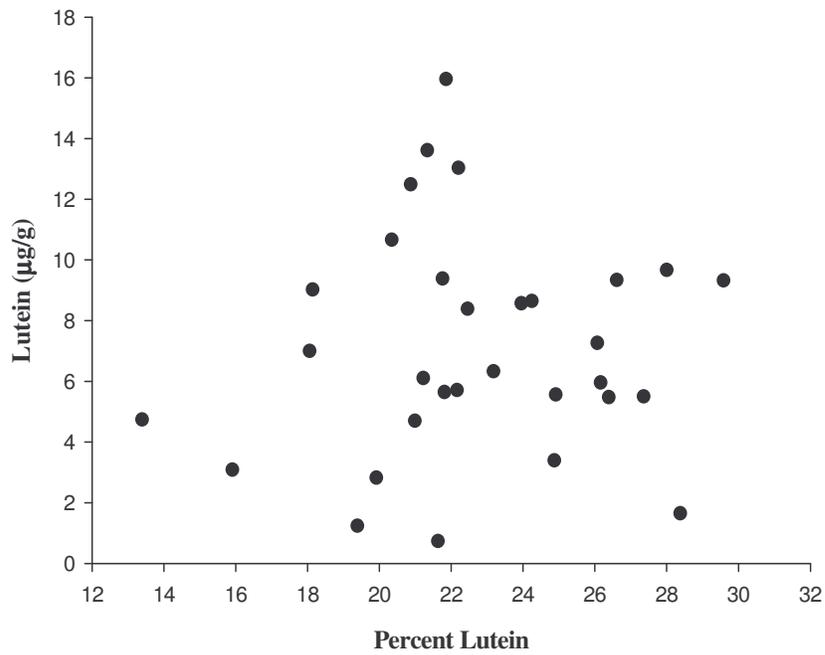


Figure 3-3. Scatterplot of percent versus concentration of lutein and β -cryptoxanthin in the eggs of coots from Eyebrow Lake, Saskatchewan, Canada.

3.4 Discussion

Within each scale of investigation, that is, among sites, among hens, and among treatments, concentrations of all individual carotenoids in the eggs of American coots were highly variable, but percentages of individual carotenoids had some consistency (Tables 3-2, 3-3, and 3-5). The role of diet in determining carotenoids in eggs has been supported from this experiment. Although several studies have demonstrated that variation in the concentrations of carotenoids in the diet can impact carotenoids in the yolk (Blount *et al.* 2002b; Royle, Surai, & Hartley 2003; Biard, Surai, & Møller 2005), here I show that under natural conditions there can be substantial variability in carotenoid concentrations among breeding locales (Fig. 3-1 and Table 3-2). This natural variability certainly lends support to arguments that some environments may be limited in their availability of carotenoids (Olson & Owens 1998).

Treatments effects on total carotenoid concentrations and for $CV_{\text{concentration}}$ further support a relationship between diet and concentration of carotenoids in yolk. Most importantly, the increase in the $CV_{\text{concentration}}$ observed in the two groups on supplemented diets substantiates the likelihood that the coots broadened their diets by feeding on both natural and supplemental food (Table 3-5). The $CV_{\text{concentration}}$ for lutein, zeaxanthin and β -carotene was similar to the $CV_{\text{concentration}}$ of canthaxanthin, β -cryptoxanthin, echinenone, and the sum of unknowns among the sites (Table 3-2), among treatments (Table 3-5) and among hens (Table 3-3); however, the proportions of canthaxanthin, β -cryptoxanthin, echinenone, and the sum of unknowns were consistent in being highly variable in contrast to a comparatively low CV_{percent} for lutein, zeaxanthin and β -carotene (Tables 3-2, 3-3, and 3-5). While it is possible that within

and among sites there was a consistent availability of a food that contained the ideal relative proportions of lutein, zeaxanthin and β -carotene, the results of my experiment support control by the hen over the percentage deposition of the suite of these three carotenoids. The food supplementation experiment successfully reduced the total carotenoid content of the coot's diet (Fig. 3-2), as well as directly manipulated lutein, and yet the relative proportions of lutein, zeaxanthin and β -carotene remained similar. Differences in $CV_{\text{concentration}}$ of lutein between the two supplemental treatments approached 35%, but differences between CV_{percent} were less than 3%. In contrast, echinenone had differences between $CV_{\text{concentration}}$ of nearly 52%, and CV_{percent} likewise was over 34%. These results suggest that some carotenoids may be transferred into the yolk as they occur in proportions in the maternal diet, and other carotenoids may be regulated in prescribed doses.

There are various explanations for why such regulation should exist. Interactions among carotenoids can influence their transfer from the diet into the body (reviewed in Surai 2002). For example, lutein can have an inhibitory effect on β -carotene absorption and assimilation (Van Den Berg 1999). Similarly, an effect of canthaxanthin concentrations on the uptake of β -carotene and lutein and zeaxanthin in tissue has been observed (Van Den Berg 1999). There is also a suite of other potential interactions between carotenoids that can either inhibit or enhance the uptake or release of carotenoids from tissues and from plasma lipoproteins. For either these or for some other physiological reason, there may have been selection for the deposition of certain proportions of carotenoids to enhance or inhibit the transfer of carotenoids from the yolk to developing embryonic tissues. Furthermore, the transfer of certain carotenoids from

the yolk to embryonic tissue appears to be controlled in that there are tissue specific proportions of individual carotenoids in the tissues of the freshly hatched chick (Tyczkowski & Hamilton 1986; Surai *et al.* 1999). These concentrations may be important for gene expression. There could also be long-term consequences of carotenoid concentrations in the egg such as effects on the ability to metabolise carotenoids in postnatal life (Koutsos *et al.* 2003).

The significance of variation in the concentrations of carotenoids among locales awaits further study. There may be trade-offs between quality and quantity of food types. A new spectrum of costs and benefits could be explored when examining questions regarding territory selection, offspring quality and parental care. How interactions between relative amounts of carotenoids in the egg and the specific carotenoids involved in immunomodulation and coloration (McGraw & Ardia 2003) have yet to be explored (Saks, McGraw, & Hőrak 2003). There could also be energetic costs associated with the maintenance of a percentage of certain carotenoids in the yolk (Blount *et al.* 2002b) and whether some of these costs could be offset by selecting certain carotenoids from the higher coefficient of variation group could be a further area of investigation.

Birds have been exploiting novel, human-altered environments that provide foods that differ in carotenoid content from natural diets, which in itself could be of concern. For example, birds such as White Storks (*Ciconia ciconia*) feed on introduced species of prey and at refuse dumps which results in variation in dietary and plasma carotenoids (Negro *et al.* 2000) and Great Tits (*Parus major*) have lower total carotenoid concentrations within the yolk when breeding in urban versus rural habitats

(Hörak *et al.* 2002). Agriculture has provided a readily available food source, such as cereal crops, for some birds and while an increase in the amount of food may seem beneficial, Eldridge and Krapu (1988) demonstrated a negative effect on the quality of eggs laid by grain-fed Mallards (*Anas platyrhynchos*) and cereal grains are known for their low carotenoid content. Despite the impact of diet on concentrations of carotenoids, because the relative percentages of the important carotenoids in egg yolk remain stable, then birds may have adapted to respond to novel environments. That is, the regulation of proportions of essential carotenoids may allow birds to exploit a variety of food types that differ in carotenoid content, of which the coot is a prime example, and yet ensure that the egg provides a nutritionally appropriate environment for embryonic development.

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CHAPTER 4
WHY DO YOLK CAROTENOID CONCENTRATIONS VARY WITH LAYING
SEQUENCE?

4.1 Introduction

Carotenoids are a group of lipid-soluble micronutrients that are synthesised by plants and photosynthetic micro-organisms. Animals ingest carotenoids and allocate them to a variety of important physiological functions. Carotenoids have been identified to perform as vitamin A precursors (Olson 1989), potent antioxidants (Surai 2002b), important correlates to immune response (Bendich 1989, Blount et al. 2003, Alonso-Alvarez et al. 2004), and as pigments (Hill 1995, McGraw et al. 2004).

In birds, carotenoids are reputed to have integral functions within the female during reproduction where they may protect vulnerable yolk precursors from peroxidation and thus increase a female's ability to produce eggs (Blount et al. 2004). Carotenoids have been long used by the poultry industry as pigments in yolk to appeal to consumers (Marusich et al. 1960), but a functional role for carotenoids in yolk has also been recognised (Blount et al. 2000). Carotenoids in yolk are absorbed by developing embryos where they function as antioxidants within the tissues (Surai and Speake 1998). There they protect vulnerable DNA, proteins, and lipids against free radicals (Surai et al. 1996, Surai 2002a) especially during hatching which is a time of extreme oxidative stress (Surai 1999). In some species, carotenoids in yolk also function to provide colour to the feathers and skin of developing chicks (Biard et al.

2005). Moreover, carotenoids in yolk have an immunostimulant role and enhance survival in resultant nestlings (Haq et al. 1996, Blount 2002, Saino et al. 2003).

The maternal deposition of carotenoids into yolk is an essential part of ensuring a nutritionally appropriate environment for the developing embryo. Although few studies have been conducted to date, a pattern is emerging with regard to how carotenoids are apportioned between successive eggs in a clutch; without exception, concentrations of these valuable nutrients decline with egg sequence. Studies of wild Barn Swallows (*Hirundo rustica*) (Saino et al. 2002), Lesser Black-backed Gulls (*Larus fuscus*) (Blount et al. 2002b), Great Tits (*Parus major*) (Hörak et al. 2002), and a group of eight passerines (Cassey et al. 2005), showed the concentration of carotenoids in the yolk declined over the laying sequence. In Red-legged Partridges (*Alectoris rufa*) (Bortolotti et al. 2003) and Zebra Finches (*Tainiopygia guttata*) (Blount et al. 2002b) in captivity, a similar decline was observed. However, a definitive answer for why such a pattern might exist has yet to be produced.

One hypothesis posed by Blount et al. (2002) suggests that the concentration of carotenoids among yolks in a clutch declines in a pre-programmed manner similar to other intrinsic strategies by female birds for differentially allocating hormones (Reed and Vleck 2001, Williams et al. 2005) and macro-nutrients (Williams 1994) into their eggs of different reproductive value. Considering that carotenoids influence immunocompetence (Bendich 1989, Blount et al. 2002b, Saino et al. 2003, Blount et al. 2003), vigour of offspring (Fenoglio et al. 2002, Blount 2002), and there appears to be some control for specific carotenoid profiles (Blount et al. 2002a, Chapter 3), it follows that observed decreases of carotenoids with laying sequence may be a strategy to

differentially invest in young (Hörak et al. 2002, Blount et al. 2002b, Royle et al. 2003, Chapter 3). Furthermore, if the pattern of decline has evolved and is associated with life history traits such as brood reduction, then it is likely that the decline should be consistent, and therefore predictable, and independent of resource levels, analogous to observed seasonal changes in carotenoid based coloration (Negro et al. 1998). I chose to call such an explanation the, “Adaptive Maternal Effect hypothesis”. However, there are other plausible hypotheses.

Royle et al. (2003) suggested that the decline in carotenoid concentrations over the laying sequence is purely a response to levels of carotenoids within the diet or within stores in the female body, and which I call here a “Carotenoid Availability hypothesis”. This explanation was supported by Bortolotti et al. (2003) who found egg laying to be a major demand on carotenoid stores in the Red-legged Partridge. Declines with egg sequence could be observed in carotenoid-limited environments when diets fail to satisfy both the female’s demand for her own needs and those of her clutch, and so reserves of carotenoids within females could be mobilised to compensate for the lack of resources. However, reserves within a female are finite, therefore the patterns of decline of the concentration of carotenoids over the laying sequence may be a response to limitations of the female to disburse the carotenoids from her diet or activate those in her stores.

My last explanation has yet to be proposed formally in the literature. The decline in carotenoids over the laying sequence could be a by-product of avian physiology, or the “Physiological artefact hypothesis”. For example, interactions between carotenoids and hormones (Verboven et al. 2005), carotenoids and very low

density lipoproteins (VLDL) (Blount et al. 2004), the ability of the hen to mobilise stores, or competitive interactions between carotenoids (reviewed in Surai 2002a) could influence carotenoid concentrations in yolk. Alternatively, the structure of the carotenoid could have an impact on the assimilation of carotenoids (During and Harrison 2004), carotenoid uptake in VLDL, or the transport or deposition of carotenoids in the yolk sac membrane. In this sense, the pattern of carotenoids *per se* is non-adaptive.

No study to date has focused on differentiating between these competing hypotheses. While preceding studies manipulating diet and examining patterns of concentrations of carotenoids in yolk over the laying sequence yielded insights, they did not mutually exclude any hypothesis. The American Coot (*Fulica americana*) has previously been used in food supplementation experiments (Hill 1988, Arnold et al. 1991). American Coots exhibit brood reduction behaviour and typically only chicks from the first half of the clutch survive to fledging (Lyon 1993). Given the latter, the Adaptive Maternal Effect hypothesis predicts that the pattern of yolk carotenoids should be a decline with sequence and be independent of diet. However, according to the Carotenoid Availability hypothesis, the pattern with sequence should vary with dietary content of carotenoids. The decline in carotenoids should only occur in situations where carotenoids are limited. As coot eggs are endowed with exceptional levels of carotenoids (see below), I take the novel approach of a carotenoid deprivation experiment and manipulate diets by reducing carotenoids, rather than supplementing them as done elsewhere (e.g., Blount et al. 2002b, Royle et al. 2003). The Physiological Artifact hypothesis predicts that coot eggs should show the same pattern of variation as

other species, and that there would be no effect of treatment. Finally, my diet experiment directly tests the Carotenoid Availability hypothesis and predicts that patterns should vary with dietary access to carotenoids.

4.2 Methods

For general methods, see section 1.3. Coots have among the highest carotenoid levels in their yolks of any bird (Surai et al. 2001); therefore, my goal was to reduce the carotenoid levels in the female's diet. In addition, I chose environments from which the carotenoid content of the eggs were low compared to other sites based on a preliminary study (fig. 4-1).

My general statistical method to examine effects on the concentrations of total carotenoids in yolk was to use analysis of covariance (ANCOVA) (PROC MIXED; SAS Institute 1999). Clutch size was limited to eggs at or below laying sequence number 10 to exclude eggs from supernormal clutches potentially caused by feeding or removing eggs (Arnold 1992, Lyon 2003, Hargitai et al. 2005). A clutch size of 10 eggs is within previously identified normal values (Hill 1988, Arnold 1990, Lyon 2003, Budden and Beissinger 2005). To standardise my feeding regimes, I generated total carotenoid values for each egg by running a general linear model for the two treatment groups at each site with time on diet as a covariate. Estimates of total carotenoids based on time on diet were normally distributed and used in subsequent analyses with total carotenoid data from control eggs. In all analyses, relevant factors, covariates and 2-way interactions were included in the model and beginning with interactions, non-significant effects were iteratively removed using a stepwise backwards elimination procedure at a

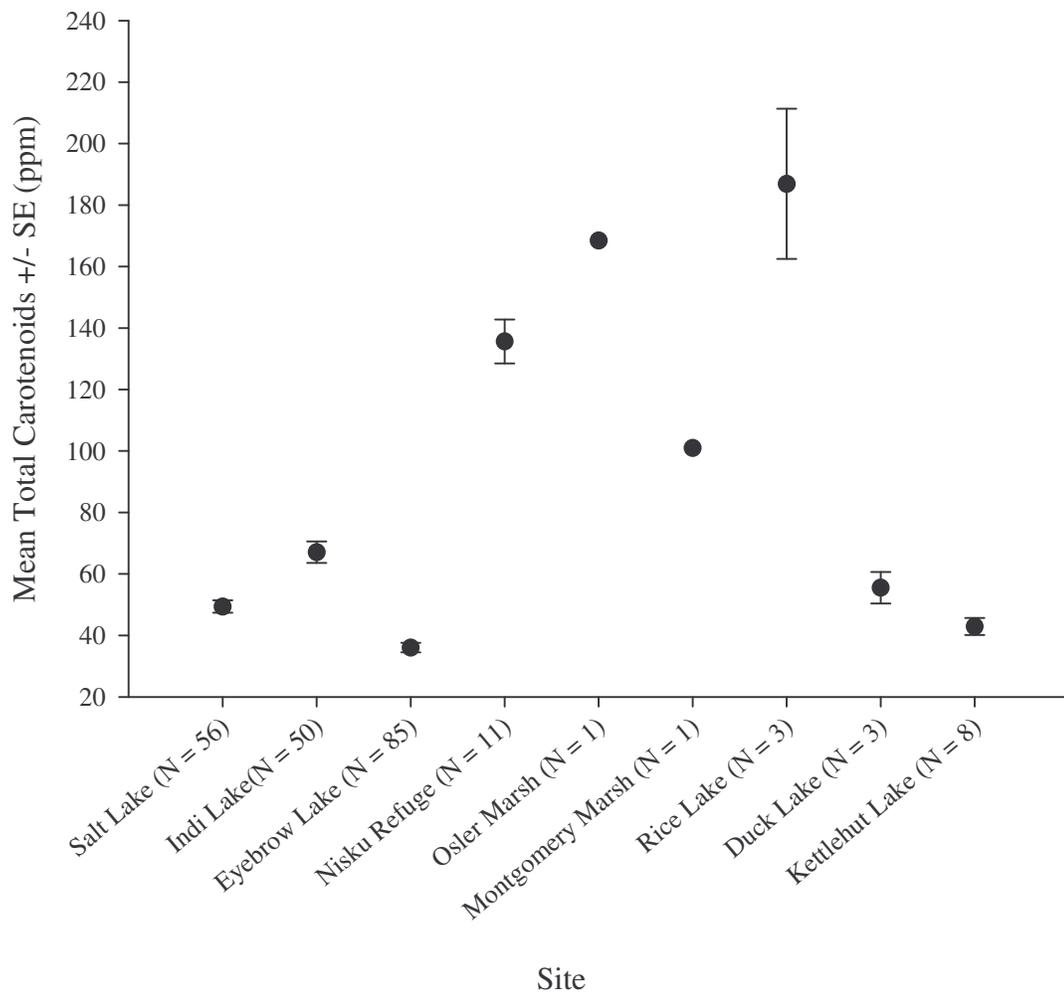


Figure 4-1: Mean \pm SE for total carotenoid concentrations of eggs of American Coots on natural diets by collection sites in Saskatchewan, Canada.

significance level set *a priori* at $p = 0.05$. I calculated degrees of freedom using the Satterthwaite method (SAS Institute 1999).

As the marshes were spatially separate and had observable variation in physical characteristics, I presumed that they should have a range of food availability. Variation in the timing of insect emergences or water plants phenology between the separate sties could potentially affect the variables that influence carotenoids in the yolk via changes in the diet. Hence, I assessed effects on carotenoids on a per site basis with the rationale that using the sites as replicates should allow me to identify any general consistencies in the variables that influence carotenoids. To examine variation of carotenoids within clutches, the ANCOVA contained the random factors of block and nest to account for potential differences within the marsh and to control for pseudoreplication caused by sampling eggs from the same hen within a nest (Fargallo et al. 2003). Treatment was inserted as a fixed factor. Covariates in the global model included position in the laying sequence, clutch initiation date, egg volume, and yolk mass.

To test for the dependency of the pattern of carotenoids over the laying sequence on resource level, I looked at whether a change in the concentration of carotenoids over the laying sequence was correlated with the concentration in the first egg within a clutch. I obtained the slope of total carotenoids over the laying sequence within each treatment group per site. I then correlated the slope with mean total carotenoid values of the first laid eggs, within each treatment group using a 2-tailed, Spearman's rank correlation.

4.3 Results

My data showed a novel pattern of an increase in the carotenoid concentration over the laying sequence, especially in the control eggs at both Salt and Indi Lakes (Salt Lake $r = .681$, $P < .001$; Indi Lake $r = .821$, $P < .001$) (fig. 4-2). Laying sequence was a significant factor in all sites (Table 4-1) but the rate of change was variable among sites and treatments (fig. 4-2). The pattern of carotenoids over the laying sequence generally showed an increase in the control groups and a stepwise decrease in the moderate carotenoid and low carotenoid diets respectively.

I successfully achieved a reduction in the carotenoid content of the eggs with my supplemented diets at all three sites (i.e. treatment, Table 4-1). Typically, the control eggs had the greatest concentration, and the moderate and low carotenoid treatments were reduced in the predicted stepwise fashion (fig. 4-2). Generally, the first eggs in the laying sequence had similar concentrations among the treatments and diverged over the clutch (fig. 4-2).

Clutch initiation date had a significant but opposite effect at Salt Lake and Indi Lakes (Table 4-1). At Salt Lake, concentrations of carotenoids in first-laid eggs increased over the reproductive period in all treatments. In contrast, the concentrations in first-laid eggs from nests in Indi Lake showed a gradual decline over the reproductive period. These patterns of concentration with clutch initiation date caused the interaction between laying sequence and clutch initiation date at both Salt and Indi Lake (Table 4-1).

It appeared that the experimental treatments had a moderate effect on egg quality parameters within Salt and Eyebrow Lakes. That is, there was a significant interaction between treatment and yolk mass at Indi Lake, where the smaller yolks from the

treatment groups had lower concentrations of carotenoids compared to the control. The interaction between egg volume and treatment at Eyebrow appeared to drive up the quality of eggs; that is, the larger the control egg, the greater the carotenoid concentration within it. In contrast, the larger the egg within the supplemented groups, the lower the concentration. Furthermore, eggs within the moderate-carotenoid diet were larger than the eggs within the low-carotenoid diet.

The rate of change with sequence differed among sites. These different rates of separation over the laying sequence among treatments at Indi Lake and Eyebrow Lake caused the interaction between laying sequence and treatment. These results, combined with the observation that the mean concentrations of first eggs varied among sites (fig. 2), led me to explore whether there was a dependency of the pattern of carotenoids over the laying sequence on resource level, that is, was the rate of sequential deposition of carotenoids (i.e. slope) a differential allocation in response to our manipulation of availability of carotenoids. However, there was no correlation between the concentration of the first egg and the rate of increase over the laying sequence ($r_s = 0.442$, $P = 0.234$, $n = 9$).

Table 4-1: ANCOVA statistics for effects on the concentration of carotenoids in the eggs of American Coots at three study sites in Saskatchewan.

Variable	Salt Lake			Indi Lake			Eyebrow Lake		
	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>	<i>F</i>	d.f.	<i>p</i>
Laying Sequence	9.04	1, 72	0.0036	5.42	1, 140	0.0213	32.43	1, 165	<0.0001
Treatment	0.0301	2, 15	0.0301	4.62	2, 139	0.0114	4.23	2, 191	0.0160
Clutch Initiation Date	16.74	1, 41.2	0.0002	24.52	1, 129	<0.0001	-	-	-
Egg Volume	-	-	-	20.42	1, 131	<0.0001	0.67	1, 187	0.4136
Yolk Mass	-	-	-	3.34	1, 3.34	0.0698	-	-	-
Laying Sequence *	8.19	1, 70.8	0.0055	6.16	1, 140	0.0143	-	-	-
Clutch Initiation Date									
Laying Sequence *	-	-	-	12.94	1, 131	<0.0001	6.76	2, 169	0.0015
Treatment									
Treatment * Yolk Mass	-	-	-	4.98	2, 140	0.0081	-	-	-
Egg Volume * Clutch									
Initiation Date	-	-	-	20.46	1, 131	<0.0001	-	-	-
Treatment * Egg									
Volume	-	-	-	-	-	-	5.00	2, 191	0.0077

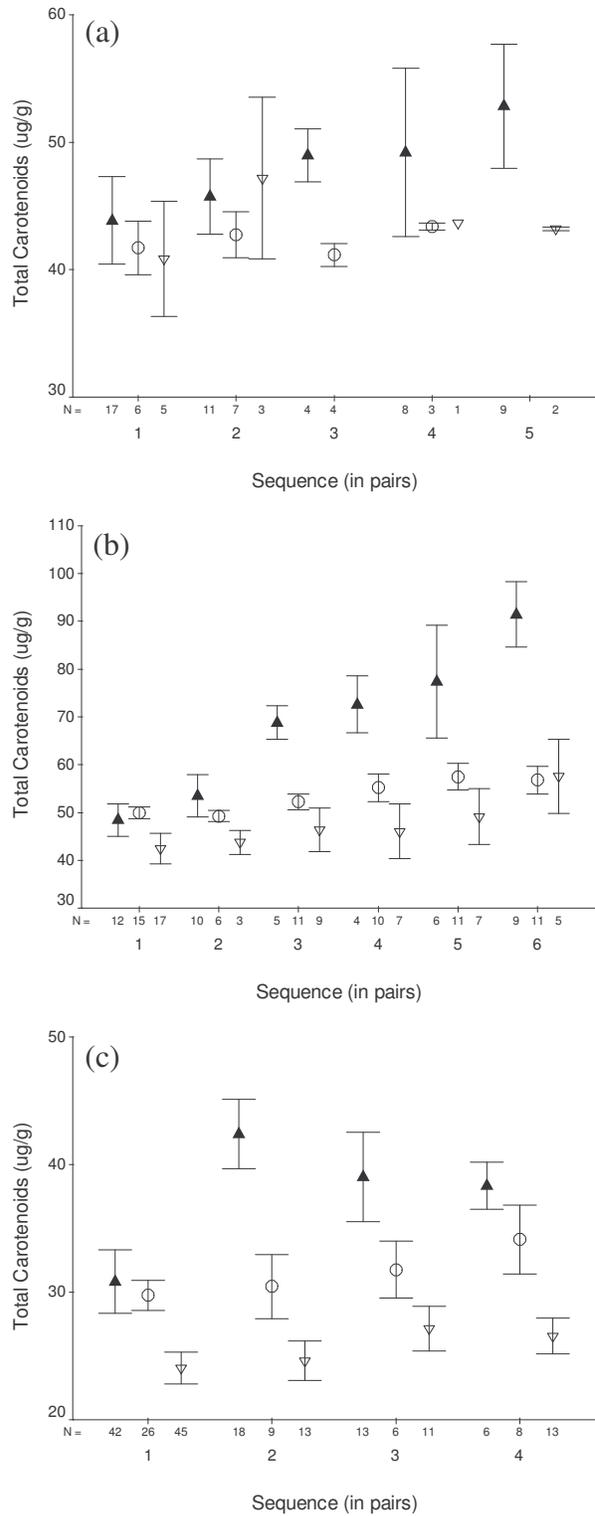


Figure 4-2: Mean total carotenoid concentration ($\mu\text{g/g}$ yolk \pm 1 SE) controlled for time on diet, of eggs laid by American coot females on (▲)control, (○) moderate, and (▽) low carotenoid diets over the laying sequence at (a) Salt Lake, (b) Indi Lake and (c) Eyebrow Lake.

4.3 Discussion

My data show that the concentration of carotenoids in the eggs of American Coots over the laying sequence depends on breeding locale and treatment (fig. 4-2). Furthermore, carotenoid content increased over the laying sequence in eggs from control nests, a pattern that is contrary to all previous studies. More importantly, the consequence of my diet manipulation was a change in the pattern of allocation of carotenoids over the laying sequence compared to the control.

Contrary to the Physiological Artifact Effect hypothesis, and unique to the American Coots, I observed an increase with laying sequence. Therefore, a commonality of avian physiology can not be responsible. Cassey et al. (2005) demonstrated that in passerines, ecological factors are more likely than phylogenetic relationships to determine the concentrations of carotenoids in eggs; here, I showed that the pattern of deposition changes with locale and carotenoid availability which should not occur if carotenoids are deposited based solely on a correlative aspect of physiology.

The pattern among treatments does not support the Adaptive Maternal Effect hypothesis. Brood reduction in American Coots dictates that only the first four chicks usually survive to fledging (Lyon 1993) and hence the value of later laid eggs should be minimal. For this reason, it is intuitive that coots should invest greater amounts of a valuable nutrient into early eggs in order to impact the fitness of nestlings. My data however, show the opposite trend; the eggs with the highest concentrations of carotenoids were observed later in the clutch (fig. 4-2). Most importantly, when carotenoid resources were manipulated, the pattern of increasing concentrations of carotenoids over the laying sequence responded by largely disappearing (fig. 4-2).

Hence, the patterns appear resource dependent, making the Carotenoid Availability hypothesis the most parsimonious explanation for the data.

There were commonalities in the significant factors within the sites that also support the Carotenoid Availability hypothesis. Treatment was significant in all sites which confirms that by altering carotenoid availability in the diet, total carotenoids in the yolk respond, which is consistent with differences observed in Great Tits and several species of passerines between habitats (Hörak et al. 2002, Cassey et al. 2005). Several of the interactions included clutch initiation date (Table 4-1). I did not measure the natural abundance or variability of natural foods within the environment; however, clutch initiation date could well correlate with site-specific changes in the availability of foods.

Carotenoids have long been hypothesized to be a scarce commodity in the environment (Endler 1980, Slagsvold and Lifjeld 1985, Hill 1990, Grether et al. 1999) but see (Hudon 1994) and my data support that some environments may be deficient. First, there was variation in the total carotenoid concentrations in eggs from different nesting locales (fig. 4-1). When I provided the carotenoid-reduced diets, the natural pattern of deposition over the laying sequence was altered. This suggests that previous studies may have supplemented carotenoids under conditions where carotenoids were limited. For example, two of the studies were under controlled conditions with artificial feeds (Bortolotti et al. 2003; Royle et al. 2003) and artificial diets are comparatively low in carotenoids compared to natural diets (Speake et al. 1999). Both Royle et al. (2003) and Bortolotti et al. (2003) were able to nearly double the concentrations in the yolk by supplementing with a diet enriched in carotenoids compared to one low in carotenoids.

Blount et al.'s (2002b) study species was a wild gull, but their successful manipulation of carotenoids in the yolk suggests that the environment in which the gulls were breeding was not saturated. When carotenoids were artificially reduced, the pattern of carotenoids in yolks over the laying sequence in American Coots was more similar to other species.

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CHAPTER 5 SYNTHESIS

5.1 Summary

While there has been much research on trade-offs between the signalling functions of carotenoids in sexual selection and their associations with health, the study of carotenoids in egg yolk is a relatively unexplored field. The deposition of carotenoids in the yolk of birds has been known since 1919 (Palmer and Kempster 1919); however, it has only been in the last few years that any significant strides have taken place in elucidating the significance of these molecules. The results of these studies have forced ecologists to collaborate with biochemists which has increased the resolution of egg quality by focussing attention beyond macronutrients into the spectrum of micronutrients.

This study was in a sense exploratory, being designed to identify associations between carotenoids in the diet and the subsequent allocation into eggs. My approach to the study was of increasing resolution; I examined the relationship between carotenoids and eggs at the level of sites, hens, and finally, within the clutch. Rather than depending on observational or correlative evidence, I used a novel, carotenoid deprivation experiment.

As I reviewed in Chapter 2, there have been inconsistencies in the reproductive response to supplemental feeding of coots and all studies have been limited to examination of the macronutrient content of the egg as an indicator of quality. Because it was necessary to understand the effects of my experimental diets on attributes of the

egg beyond those of merely concentration of carotenoids, I used my feeding experiment to fulfill two objectives. The data produced for chapter 2 provide an explanation for the variable responses to supplemental feeding experiments involving the size of eggs in coots and they demonstrate a new tool for measuring the quality of diet of females. First, I use stable isotopes to demonstrate that it is, in fact, the local diet (i.e. exogenous sources of food) that is most affecting the size of eggs. Then by providing an easily obtainable source of nutrients, by manipulating carotenoids in the diet, and by measuring carotenoids in eggs, I show that it is diet quality and not quantity of food that has a significant effect on the size of eggs.

The use of carotenoids as an indicator of egg quality has been proposed (Royle et al. 2003), but their use of an indicator of diet quality has not. Considering that carotenoids are essential nutrients and may be limited, suggesting that a greater concentration of carotenoids in yolk is indicative of diet quality is reasonable. Furthermore, my correlative evidence associating carotenoids and $\delta^{13}\text{C}$ also support the use of carotenoids as an indicator of dietary quality.

In chapters 3 and 4, I delve into the relationship between nesting locales, diet and the allocation of carotenoids to eggs. There has been debate on whether the deposition of carotenoids is adaptive, where females control the apportioning of carotenoids into egg yolk or if females are merely responding to availability of carotenoids in the environment or their bodies. In chapter 3, I show that the allocation depends on the type of carotenoid of interest. Among my 3 levels of exploration, that is, among sites, among hens and among treatments, the percentages of 3 important carotenoids (lutein, zeaxanthin and β -carotene) appear regulated. However, the

remaining individual carotenoids are being deposited in percentages that depend on the particular environment where the bird is living. I also show that there is variation in the individual carotenoid concentrations among sites and in chapter 4, I report that the difference in total concentration in eggs among 8 sites can be up to an order of magnitude among sites.

Chapter 4 examined factors that could influence the total concentrations of carotenoids within eggs, with particular emphasis on the patterns associated with laying sequence. In a dozen species of birds the pattern of deposition of carotenoids over the laying sequence has consistently been that of a decline (Hörak et al. 2002, Saino et al. 2002, Blount et al. 2002, Royle et al. 2003, Bortolotti et al. 2003, Cassey et al. 2005). Two hypotheses (Adaptive Maternal Effect and Carotenoid Availability) have been suggested and most support has been for the Adaptive Maternal Effect hypothesis (Blount et al. 2002, Hörak et al. 2002, Royle et al. 2003, Cassey et al. 2005). In chapter 4, I pose a new hypothesis (Physiological Artifact hypothesis) and test all three hypotheses with a new species. Several important conclusions resulted from aspects of this study. Most importantly, the pattern with laying sequence varied with availability of carotenoids in the diet. Common factors that were significant at all sites were those of sequence and treatment which also correlate with the availability of carotenoids in the environment. Coots also showed a unique pattern of increase in carotenoids over the laying sequence in the control eggs which excluded the Physiological Artefact hypothesis and was contrary to the Adaptive Maternal Effect hypothesis. Hence, my data support the Carotenoid Availability hypotheses where the availability of carotenoids influences the patterns of allocation over the laying sequence.

5.2 Implications and Future Directions

The results of my work provide insight on the ecophysiology of carotenoids in birds and suggest new tools for assessing the quality of environments. For example, chapter 2 shows that carotenoids are correlated with dietary attributes that influence the size of eggs. By collecting eggs from birds that rely on exogenous nutrients for egg formation from different sites, managers can assess the quality of habitats. In a species relying on endogenous sources of nutrients for egg formation and if the wintering area of a bird is known, carotenoids in the yolk can provide an indication of environmental quality of wintering grounds and migration routes. Furthermore, current research has shown that the colour of yolk correlates with the concentration of carotenoids in eggs which could make it an effective tool in the field.

An understanding of the consequences of carotenoids in eggs is required. My work demonstrates that there are differences among locales, among hens, and between experimental treatments; however, how these differences affect fitness remains to be elucidated. For example, my results in chapter 3 suggest that some carotenoids are merely allocated into the egg as a consequence of diet. Whether these carotenoids can be converted or utilised to compensate for reduced availabilities of the 3 major carotenoids, lutein, zeaxanthin and β -carotene, is unknown. There may also be trade-offs with adult health and carotenoid deposition which could only be examined with assessments of female stores over the laying period.

Thresholds to minimal concentrations of carotenoids in egg yolk are poorly understood. Are there minimal amounts of carotenoids or certain individual carotenoids that are necessary to boost immune system development in the embryo? Are there

species specific thresholds necessary to provide certain functions? For example, the bright coloration of the plumage of chicks of coots may mean that the threshold for concentrations of carotenoids in coots is higher than other birds. Further, birds with differing breeding strategies (i.e. precocial versus altricial), may have different requirements for carotenoids. There may be subtle effects on behaviour and fluctuating asymmetry that could have long reaching consequences to the fitness of individuals.

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