

Exploratory behavior is associated with plasma carotenoid accumulation in two congeneric species of waterfowl



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ARTICLE INFO

Article history:

Received 14 November 2014

Received in revised form 27 March 2015

Accepted 17 April 2015

Available online 18 April 2015

Keywords:

Activity

Anas

Carotenoid pigments

Mallard

Northern pintail

Personality

ABSTRACT

Recently, carotenoid pigments have received considerable attention as modulators of animal health and performance. While studies show that elevated carotenoid intake and accumulation can influence activities like parental care and escape-flight performance, little is known of how carotenoid status influences the expression of animal personality traits, which can be energy-demanding and entail survival costs but also rewarding in the context of foraging and mating. We experimentally investigated the effects of carotenoid availability on exploratory behavior and activity level, using adult males and females of two species of waterfowl: mallard (*Anas platyrhynchos*) and northern pintail (*Anas acuta*). We assessed behavior using a novel-environment test designed to measure an individual's response to novel objects and a potential predator threat (fox urine scent). We found that carotenoid availability was positively associated with some aspects of exploratory behavior: birds with higher concentrations of circulating carotenoids entered the test arena sooner and approached and entered predator-scented bedding material more frequently than birds with low carotenoid concentrations. These results suggest that the availability of carotenoid resources can influence personality traits in waterfowl, and we discuss putative physiological mechanisms underlying this effect.

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1. Introduction

In many species, individuals exhibit consistent differences in a range of behavioral traits (e.g., boldness, aggressiveness, and exploration). Furthermore, such traits are frequently correlated (Verbeek et al., 1996; Dingemans et al., 2007; Kortet and Hedrick, 2007) and are therefore regarded as aspects of an individual's personality (Gosling, 2001; Réale et al., 2007, 2010; Stamps and Groothuis, 2010). Exploratory behavior (i.e., an individual's reaction to new environmental situations; Réale et al., 2007) is a commonly measured personality trait that has been studied in a range of captive and wild organisms (e.g., Verbeek et al., 1994; Dingemans and de Goede, 2004; Carere et al., 2005; Boon et al., 2007; Dingemans et al., 2007; Jones and Godin, 2010; Butler et al., 2012). Animals may benefit from acquiring information about their environment that permits them to make quick, informed decisions about mate choice,

habitat or food selection and predator avoidance, and one way to gather such information is exploration. However, while exploratory behavior can be rewarding in the context of foraging and mating, it can also be energy-demanding and entail survival costs. As individuals vary considerably in their propensity to approach and explore new situations or objects (Verbeek et al., 1994), recent studies have aimed at understanding the mechanisms driving inter-individual variation in exploratory behavior (e.g., Carere et al., 2005; Careau et al., 2008; Butler et al., 2012). These studies have shown that exploratory behavior is heritable (Dingemans et al., 2002), with further variation driven by a range of environmental factors, including current abiotic/biotic conditions, prior and early life experience and social context (Dingemans et al., 2004; Carere et al., 2005; Schuett and Dall, 2009; Biro and Stamps, 2010; Ward, 2012).

Among the possible environmental factors contributing to variation in exploratory behavior, the nutritional status of individuals (e.g., diet quantity and composition) remains relatively understudied. Moreover, the few studies that have examined the influence of diet on personality traits more generally have focused on nutritional conditions during ontogeny. For example, nutritional deprivation and diet deficiencies early in life have been shown to

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influence exploratory behavior in a range of taxa, including birds (*Parus major* (Carere et al., 2005); *Taeniopygia guttata* (Krause et al., 2009)), fish (*Kryptolebias marmoratus* (Edenbrow and Croft, 2012)), rats (*Rattus norvegicus* (Fraňková and Barnes, 1968; Weinberg et al., 1980; Enslin et al., 1991)), mice (*Mus musculus* (Ishii et al., 2005)) and monkeys (genus *Cebus* (Elias and Samonds, 1974)). In contrast, while there is good reason to expect a link between adult nutritional status and an energy-demanding activity like exploration, investigations into the influence of variation in adult dietary intake on an individual's propensity to explore new environments are lacking. Moreover, attempts to tie particular nutrients to aspects of exploratory behavior are limited.

Carotenoid pigments have received considerable attention of late as dietary modulators of animal health and performance (Vinkler and Albrecht, 2010). In addition to their provitamin activity and role in pigmentation, carotenoids can serve as immunostimulants (e.g., Blount et al., 2003; McGraw and Ardia, 2003). Carotenoids may also serve as antioxidants by mitigating oxidative damage through the quenching of free radicals and break down of lipid peroxidation chain events (Burton and Ingold, 1984; Krinsky, 1989; Miki, 1991; Krinsky, 1998); though the importance of carotenoids relative to other antioxidant molecules (e.g., vitamins) remains unclear (Hartley and Kennedy, 2004; Costantini and Møller, 2008) and carotenoids can become pro-oxidants under certain conditions (Palozza et al., 2003; Hurst et al., 2005). Importantly, elevated carotenoid intake has been found to enhance various animal behaviors, including reproduction (Blount et al., 2004), parental effort (Pike et al., 2007; Ewen et al., 2008), song output (Van Hout et al., 2011) and escape-flight performance (Blount and Matheson, 2006). Because high carotenoid resources are associated with enhanced immunocompetence, antioxidant activity and health of individuals in a range of species (e.g., Blount et al., 2003; McGraw and Ardia, 2003 but see Costantini and Møller, 2008), including mallards (*Anas platyrhynchos* (Butler and McGraw 2012)), we hypothesized that exploratory behavior and activity level may be influenced by the availability of carotenoid resources. More specifically, since activity-induced increases in reactive oxygen species (ROS) production can lead to muscle fatigue (Powers et al., 2004), we suggest that carotenoids may influence physiological performance at the behavioral level if they provide antioxidant protection to muscles (Ji, 1999; Powers et al., 2004) or other body tissues relevant to animal movement (e.g., eyes, brain). Additionally, since recent studies have linked the past or current immune status of individuals to exploratory behavior and activity level (Butler et al., 2012; Grindstaff et al., 2012; Männiste et al., 2013), we suggest that high levels of circulating carotenoids may also promote exploration and activity behavior via their effects on overall immunocompetence or general health.

In some of the only comparable literature, (Costantini et al., 2008) found that another personality trait in mice (i.e., aggressiveness) was associated with serum antioxidant status. Similarly, plasma antioxidant capacity was positively associated with greater exploration in European greenfinches (*Carduelis chloris* (Herborn et al., 2011)). Neither of these studies, however, singled out the potential contributions from carotenoids. Here, we investigated the effect of carotenoid availability on exploratory behavior and activity level in two congeneric species of dabbling duck – mallard and northern pintail (*Anas acuta*). We selected a phylogenetically matched pair of species, where one taxon exhibits carotenoid-based integumentary coloration and the other does not, because it allowed us to examine whether differing carotenoid allocation strategies are associated with different behavioral responses. Male mallards in nuptial plumage have a yellow, carotenoid-based bill (Butler et al., 2011), whereas female mallards exhibit less colorful bills (predominately brown with some orange coloration). In contrast, neither male nor female northern pintails display

carotenoid-based integumentary coloration. Female mallards prefer to mate with males having yellower bills (Omland, 1996), whereas female pintails show a preference for males with whiter breast coloration and more colorful scapular feathers (Sorenson and Derrickson, 1994). The species and sexes also show differences in general behavior: pintails are generally considered to be largely nonaggressive and sociable, and appear to be subordinate to mallards when foraging (Bailey and Batt, 1974). Conversely, mallards tend to be more aggressive, in both feeding and mating contexts, relative to other waterfowl species (Bailey and Batt, 1974), including a congener, the American black duck (*Anas rubripes* (Brodsky and Weatherhead, 1984)). Additionally, in both species, males tend to be more aggressive than females (Bailey and Batt, 1974; Hepp and Hair, 1984), and at least in pintails, males spend more time moving relative to females (Kaminski and Prince, 1981).

We generated predictions related to carotenoid-dependent behavioral responses within two alternate frameworks linked to variation in carotenoid ornamentation and physiology. We also predicted a number of general species- and sex-specific differences in exploratory behavior and considered how these predictions may be modulated by each of the physiological frameworks. Our first framework was based on the assumption that the investment of carotenoids into integumentary coloration reduces carotenoid availability for other functions (sensu carotenoid trade-off hypothesis; Lozano, 1994). Within this framework, and under the assumption that carotenoids enhance immune function or reduce oxidative stress (see Butler and McGraw, 2012 for evidence in mallards), we predicted that birds with greater carotenoid resources (i.e., higher concentrations of circulating carotenoids) would be more exploratory and more active than those with low carotenoid levels. Moreover, given that pintails, unlike mallards, do not invest in carotenoids into integumentary coloration, we predicted that pintails with high carotenoid levels might exhibit relatively greater increases in exploratory behavior and activity level compared to mallards. Similarly, we predicted that female mallards might benefit more from high carotenoid resources than male mallards because females exhibit less colorful bills (and thus, for a given carotenoid level, should have comparatively more resources available in the body for physiological functions).

Given the biology of these two species and because of aggressiveness and exploratory behavior are correlated in a range of species (Verbeek et al., 1996), we generally expected pintails to be less exploratory than mallards due to their less aggressive nature. Similarly, we expected the typically more aggressive males of both species to be more exploratory and show greater activity levels relative to females. However, within the framework of reduced carotenoid availability when integumentary coloration is carotenoid-dependent, we expected these species- and sex-specific differences might be reduced. This is because carotenoid resources would be more available to pintails (male and female) and female mallards as these groups lack carotenoid-dependent ornamentation, and these additional resources would remain in circulation and thus be available for boosting activity levels and exploratory behaviors.

Our second framework is built on the assumption that birds displaying carotenoid-based coloration possess physiological adaptations that allow them to absorb or accumulate more carotenoids (McGraw, 2005). Under this framework, it is expected that individuals with high carotenoid resources would exhibit increases in both exploratory behavior and activity level, but that mallards would show relatively greater increases in behavioral responses compared to pintails. Following this same logic, male mallards were expected to show the greatest increases in exploratory behavior and activity level when carotenoid-replete, as they exhibit the most elaborate form of carotenoid-based coloration compared to pintails and female mallards. Finally, in terms

of our species- and sex- specific predictions (i.e., mallards more exploratory than pintails, and males more active and exploratory than females), we expected these differences might be more pronounced under the second framework. In this instance, we expected species- and sex differences might increase because male mallards are predicted to have higher circulating carotenoid levels relative to pintails and female mallards, and thus, these additional carotenoid resources would boost any innate differences in personality driven by general biology.

2. Methods

2.1. Acquisition and maintenance of birds

We acquired adult male and female mallards and northern pintails (60 mallards, 61 pintails) from commercial waterfowl breeders (Metzer Farms, Gonzales, CA; Gary Konzer Farms, Avon, MN) during April and May 2009. All birds were recently descended from wild-caught individuals and were maintained under natural or semi-natural conditions prior to acquisition. Throughout the study, animals were housed in large aviaries ($\sim 4.7 \times 1.5 \times 2.5$ m) located on the campus of Arizona State University in Tempe, AZ (USA). Each pen consisted of an indoor area ($\sim 1.2 \times 1.5 \times 2.5$ m) supplied with aspen wood shavings as bedding, and a larger outdoor section ($\sim 3.5 \times 1.5 \times 2.5$ m) that included a swimming pond (~ 1.5 m diam). Food and water were provided ad libitum throughout the study. In all instances, food and water was provided at multiple locations in the aviary; this was done to minimize any potential competition over access to food and water and to ensure that all individuals received adequate resources. All procedures undertaken in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Arizona State University (protocol number 08-979R).

Animals were maintained in single-sex, single-species groups of 15 individuals ($n = 16$ for one group) from the time of acquisition (3rd April and 1st May pintails and mallards, respectively) until 20th June 2009. During this time, all birds were fed a control diet (containing $< 3 \mu\text{g g}^{-1}$ xanthophyll carotenoids, Mazuri Waterfowl Maintenance Diet, St. Louis, MO) to minimize, but not pharmacologically eliminate, carotenoids stored in body tissues. On 21st June 2009, birds were randomly assigned to one of six dietary treatment groups and redistributed into mixed-sex, mixed-species flocks of 20 individuals ($n = 21$ for one group). Groups were rotated into a new aviary every 8–10 days to minimize the potential effect of group location. Birds were habituated to daily human presence in their aviaries and accustomed to regular capture.

For the remainder of the study, birds were fed one of six dietary carotenoid treatments, including the control diet above and carotenoid-supplemented diets containing 5, 10, 25, 50 or $100 \mu\text{g g}^{-1}$ of xanthophyll carotenoids. Dietary treatments with 5, 10, 25 and $50 \mu\text{g g}^{-1}$ of carotenoids fall within the range of carotenoid concentrations found in wild duckling diets (Butler and McGraw, 2010), while the dietary treatment of $100 \mu\text{g g}^{-1}$ of carotenoids likely represents an extreme high carotenoid dose. We chose this approach because we wanted to not only test the idea that carotenoid availability affects avian personality traits, but also to determine whether such behaviors are affected in a dose-dependent manner (i.e., is there a level of carotenoid supplementation beyond which further increases in carotenoids do not translate into additional increases in behavioral traits).

All supplemental diets were made by homogeneously coating food pellets with a mixture of OroGlo 20 carotenoid supplement (Kemin Industries, Des Moines, IA) and sunflower oil (87:13 food–oil mix). The control diet was coated in sunflower oil only. To prevent carotenoid degradation and ensure consistency of dietary

carotenoid supplementation, all diets were mixed twice weekly, partitioned into daily rations and stored in the dark at -20°C . Moreover, food was completely replaced on a daily basis, and feeding bins were opaque to prevent light exposure. All birds were maintained on experimental diets throughout the experiment. Because our experiment was conducted according to the molt schedule of individuals (see below), the duration of carotenoid supplementation varied from 22 to 32 weeks. However, in both species, circulating carotenoid levels remained stable over the duration of the experiment (REML GLMM: mallard, $F_{1,212} = 1.60$, $P = 0.21$; pintails, $F_{1,218} = 0.96$, $P = 0.33$), suggesting that the variation in the duration of carotenoid supplementation should not affect our results.

We collected whole blood from all individuals in order to measure circulating carotenoid titers. Blood samples were collected from the brachial vein using heparinized capillary tubes at the end of the carotenoid depletion period (i.e., immediately prior to carotenoid supplementation; 20th June), following 13 weeks of carotenoid supplementation and at the end of the experiment. Immediately after collection, blood was placed in an Eppendorf tube and placed on ice (for up to 6 h) before being centrifuged for 2 min at 10,000 r.p.m. The resulting plasma samples were then stored at -80°C until later analysis.

2.2. Plasma carotenoid concentration

We used high-performance liquid chromatography (HPLC) to quantify the concentration of carotenoids in plasma following McGraw et al. (2008). Briefly, we added $200 \mu\text{l}$ of ethanol to $20 \mu\text{l}$ of plasma and vortexed the solution for 5 s. We then added $200 \mu\text{l}$ of a 1:1 solution of hexane:methyl *tert*-butyl ether to the tube and vortexed the sample for another 5 s. Next, we centrifuged the solution at 12,000 rpm for 1 min and transferred the resulting supernatant to a fresh tube. The supernatant was then evaporated to dryness under nitrogen. Finally, the pigment residue was reconstituted in $200 \mu\text{l}$ of methanol:acetonitrile:dichloromethane (42:42:16, v/v/v) for HPLC analyses (sensu McGraw et al., 2006) using a Waters Corporation (Milford, Massachusetts) Alliance HPLC system equipped with a Carotenoid C-30 column (Waters Corporation). Carotenoids were identified by comparing retention times and light-absorbance maxima to authentic carotenoid standards, and the concentration of each carotenoid type was determined using external standard curves. Lutein and zeaxanthin were the only carotenoid types found in every individual, and on average these two carotenoids made up $\sim 99\%$ of the total carotenoid titer (range 92.4–100%). In addition, we found two lutein derivatives in a considerable proportion of samples, while smaller amounts of β -cryptoxanthin were detected infrequently. Circulating carotenoid concentrations were calculated as the sum of all carotenoid concentrations in plasma ($\mu\text{g ml}^{-1}$).

2.3. Behavioral trials

We conducted behavioral trials on 100 individuals (27 male and 22 female mallards, 27 male and 24 female pintails) between 21st November 2009 and 25th January 2010. Timing of behavioral trials was variable because trials were conducted according to the molt schedule of individual birds, rather than by date. Specifically, trials were run once birds were near the completion of molt into nuptial plumage (i.e., c. 50% of feathers were fully developed feathers in nuptial plumage or pinfeathers). There was no bias in testing date among experimental groups (i.e., test date was not dependent on experimental grouping; $F_{5,94} = 0.61$, $P = 0.69$).

All trials were conducted using a standard novel environment test (Verbeek et al., 1994; Dingemans et al., 2002). Specifically, trials were performed in an outdoor, non-aquatic test arena that was

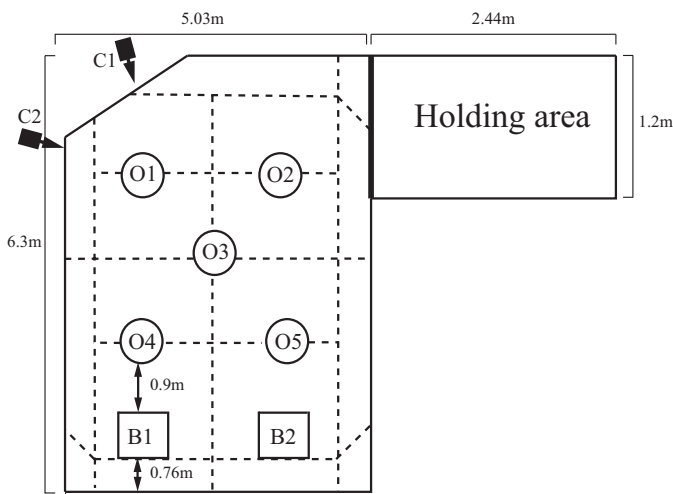


Fig. 1. Overhead schematic view of the outdoor novel test arena.

Birds entered the test arena (6.3 m × 5 m × 2.4 m) from the holding area. All walls were covered with black tarp from ground level to a height of 1.2 m to visually isolate the test arena from the rest of the colony. The test arena contained five novel objects (planting pots) placed at the center of a paint circle (ca. 60 cm diameter) drawn on the floor of the arena (O1–O5). Additionally, two trays containing aspen bedding (B1 and B2) were placed at one end of the arena. For the purposes of data collection on spatial location, the test arena was divided into 18 squares (indicated by dashed lines); divisions between sections were visually estimated by observers during data collection using the relative size of ducks, i.e., distances were approximated in duck lengths (distance from beak tip to tail tip). Two cameras (C1, C2) were mounted on the outside of the test arena to provide a full view of the test arena.

visually, but not acoustically, isolated from the rest of the population (6.4 m × 5 m × 2.4 m; Fig. 1). While both species are mostly aquatic, dabbling ducks regularly forage and nest on land (Austin and Miller, 1995; Drilling et al., 2002); thus an outdoor, non-aquatic test arena represents a biologically relevant environment. Furthermore, dabbling ducks are known to show natural and complete social behavior in captivity (McKinney, 1981), making them an ideal taxon for investigations of exploratory behavior and activity under lab conditions. The test arena incorporated several novel objects, including five planting pots painted white (Gloss White, #1992830; Rust-Oleum Painter's Touch, Vernon Hills, IL) and two trays of aspen bedding (0.6 m × 0.6 m). Planting pots were painted white to avoid any color-dependent exploration behavior (Butler et al., 2012), and each pot was placed at the center of a circle (ca. 60 cm diam) drawn on the floor of the arena in black paint. These paint circles allowed us to objectively define instances of interactions with novel objects, because individuals within that circle were deemed to be close enough to touch the object. Prior to each trial, we placed a cotton ball soaked with ten drops of red fox (*Vulpes vulpes*) urine (Red Fox Urine, Wildlife Research Center) in one randomly chosen bedding tray to assess exploration of a risky novel object (i.e., exploration in the presence of a potential predator). The red fox is considered the primary predator of both species in the wild (Sargeant et al., 1984). Although no previous literature indicated the appropriate amount of urine to use, we choose this amount because it was detectable by odor, at least to humans, at a distance of one meter.

All trials were run after sunrise, between 0700 and 1100 h; a period of peak activity for wild and captive birds of both species (MR, personal observation). Importantly, focal birds did not have any experience with the test arena prior to the trial. All trials were videotaped with two digital camcorders (Canon ZR830, Canon USA, Inc., Lake Success, NY, USA; JVC Everio, JVC USA Inc., Wayne, NJ, USA) mounted to provide a full view of the test arena. Before each trial, we removed all debris and fecal matter from the floor of the arena to ensure that no fecal cues influenced the behavior of subsequent birds. Commonly, several birds were eligible for behavioral

trials on the same day; thus, an individual randomly selected from the pool of eligible birds was captured from its home pen, transferred to the small holding area adjacent to the test arena (see Fig. 1) and left to acclimate for two minutes. Following this period, the door to the test arena was remotely opened, allowing the focal bird to enter and explore the test arena in isolation for a total of 20 min. If an individual did not leave the holding area after five minutes, one of us (KLP) approached the area to induce its exit. Birds forced to leave the holding area exhibited similar levels of exploration and activity as birds that left the holding area on their own (all $P > 0.19$, except for time spent interacting with the scented bedding, where $P = 0.003$). We therefore considered these birds as exploratory and included them in all subsequent analyses. At the end of each trial, birds were returned to their home pens and both outdoor temperature and percentage of cloud cover were recorded.

Videos were analyzed at a later date by one of us (KLP) blind to experimental diet treatment. All interactions with novel objects, both in terms of latency to approach and time spent interacting with, were recorded; an interaction was defined as the duck being within the paint circle, regardless of body orientation or travel direction. Additionally, instantaneous scan sampling at 20-s intervals was performed to quantify both the location of the focal individual within the arena and whether the individual was active (e.g., flying, running, and walking) or inactive (e.g., standing, sitting). We quantified exploration as: (a) latency (in seconds) to leave the holding area (Jones and Godin, 2010). When individuals were induced to leave the holding area latency was recorded as 301 s. (b) Number of exploration squares visited, where squares were defined by dividing the test arena into sections (Kurvers et al., 2009; Jones and Godin, 2010); see Fig. 1). (c) Number of different novel objects visited (each object included only once; Verbeek et al., 1994; Schuett and Dall, 2009). (d) Time (in seconds) taken to visit the first novel object once out of the holding area (Verbeek et al., 1994). (e) Time (in seconds) taken to visit a second novel object once out of the holding area (Verbeek et al., 1994). (f) Total time (in seconds) that individuals spent engaged in exploration of novel objects. (g) Total number of times that an individual approached the scented bedding. (h) Total time (in seconds) spent interacting with the scented bedding (defined as all instances of direct contact with the aspen bedding). Additionally, we quantified activity level as: (a) proportion of time spent active after leaving the holding area (Wilson and Godin, 2010), and (b) total number of transitions between different exploration squares in the test arena.

The amount of time that individuals spent in the test arena varied by their latency to leave the holding area. Thus, several variables were expressed as rates, including number of squares visited (i.e., area visitation rate), number of objects visited (i.e., object visitation rate) and number of times the scented bedding was approached (i.e., scented bedding approach rate). Additionally, both the total time engaged in exploration of novel objects and total time spent interacting with the scented bedding were expressed as the proportion of time spent in each activity relative to the total amount of time spent in the test arena.

Next, in order to compare behavioral responses to the scented and unscented bedding trays, we also quantified the total number of times that an individual approached the unscented bedding tray and the total time (in seconds) spent interacting with the unscented bedding (defined as all instances of direct contact with the aspen bedding); these measures mimicked those used for scented bedding in the assessment of exploration (see g and h above). As before, the number of times the unscented bedding was approached was expressed as a rate and the amount of time spent interacting with the scented bedding was expressed as a proportion of the total time spent in the test arena in order to account for inter-individual variation in time spent in the test arena. Finally, we recorded body mass at the end of the experiment, and used this value in all analyses

presented here. We used this approach because all behavioral trials were conducted within 14 days of this measurement, and because individual mass at this time was correlated with mass throughout the experiment (all $r > 0.9$, all $P < 0.0001$).

2.4. Statistical analysis

To ensure that treatment groups did not differ in plasma carotenoid levels or body mass before the start of the experiment, we used a one-way ANOVA to compare values among the dietary treatment groups at the end of the depletion period (i.e., immediately prior to carotenoid supplementation), running separate models for each species. To confirm that carotenoid supplementation elevated circulating carotenoid titers, we tested for an effect of diet treatment on plasma carotenoid levels following 13 weeks of dietary supplementation using linear models including the factors carotenoid dose and sex, as well as their two-way interaction. Models were run separately for each species and the interaction term was removed if not significant (*sensu* Zuur et al., 2009). Next, focusing on carotenoid values observed at the end of the experiment (i.e., those observed at the time of behavioral trials), we tested for an effect of diet treatment on plasma carotenoid levels using linear models. As before, predictor variables included carotenoid dose and sex, as well as their two-way interaction, and models were run separately for each species and the interaction term was removed if not significant. Additionally, we compared circulating carotenoid levels among the dietary treatments using pairwise comparison tests, separately for each sex and species, implemented in the R package 'lsmeans'. This function returns adjusted P values for all pairwise comparisons of means using the Studentized range distribution with the number of means in the family (i.e., Tukey's adjustment).

Because several measures of exploratory behavior were inter-correlated (Table 1), we used a principal components analysis (PCA) with varimax rotation, scaled and centered to zero, on all eight measures of exploratory behavior to reduce the number of parameters to a limited number of synthetic variables. Similarly, we used PCA to reduce measures of activity to a limited number of variables for subsequent analysis. Only extracted axes with eigenvalues > 1 and clear biological interpretations were retained for further analysis, and behaviors with high loadings were considered to contribute to the meaning of the component (see Section 3 and Table 2 for details of the retained PCs).

We then used GLS regression to examine predictors of behavior PCs. In all models, we tested the effect of plasma carotenoid

Table 2

Results of the principal components analyses for (a) exploratory behavior and (b) activity level.

	PC1	PC2
(a) Exploratory behavior		
Latency to leave holding area	−0.084	−0.695
Time to first object	−0.466	−0.004
Number of squares visited (i.e., area visitation rate)	0.188	0.153
Numbers of objects visited (i.e., object visitation rate)	0.535	−0.086
Time to second object	−0.444	0.233
Time spent exploring objects	0.500	0.008
Scented bedding approach rate	0.059	0.455
Time interacting with scented bedding	0.062	0.474
Cumulative proportion of variance explained	40.3	54.7
Eigen values	3.23	1.15
(b) Activity level		
Time spent active	0.707	
Transition rate	0.707	
Cumulative proportion of variance explained	93.98	
Eigen values	1.88	

Shown are PCA loadings, with contributing factors in bold, and the cumulative proportion of variance explained by (a) the first two principal components extracted by PCA of 8 measures of exploratory behavior, and (b) the first principal component extracted by PCA of 2 measures of activity level in adult male and female mallard and pintail ducks.

concentrations. Despite our initial aim of examining potential dose-dependent effects of carotenoid supplementation, we chose to examine the effect of circulating carotenoid levels and not dietary carotenoid dose per se for two reasons. First, due to last-minute logistical constraints of animal housing, we were unable to hold birds in individual cages and instead birds were held in groups that reflected dietary carotenoid level treatment. Consequently, individuals within a treatment group were not independent, and thus, analysis of the effect of carotenoid dose would suffer from pseudo-replication. More importantly, although carotenoid supplementation enhanced circulating carotenoid levels, these effects did not reflect the underlying dietary doses. More specifically, low carotenoid doses (i.e., 5 and 10 $\mu\text{g g}^{-1}$ carotenoids) did not necessarily elevate circulating carotenoid levels above control levels. Furthermore, at high carotenoid doses (i.e., 25, 50 and 100 $\mu\text{g g}^{-1}$ carotenoids), there was considerable variation and overlap in circulating carotenoid levels, likely due to individual variation in carotenoid assimilation ability, such that carotenoid titers frequently did not differ between treatment groups (see results below for further information). Thus assessing carotenoid availability in

Table 1

Correlations among the eight exploratory behavior measures quantified in the test arena.

	Time to 2nd Object	Number of objects visited	Number of squares visited	Time to 1st Object	Latency to leave holding area	Scented bedding approach rate	Time interacting with scented bedding
Time spent exploring objects	$r_s = -0.68$ $P < 0.0001$	$r_s = 0.97$ $P < 0.0001$	$r_s = 0.22$ $P = 0.09$	$r_s = -0.87$ $P < 0.0001$	$r_s = -0.11$ $P = 0.38$	$r_s = 0.12$ $P = 0.38$	$r_s = 0.12$ $P = 0.38$
Time to 2nd object		$r_s = -0.74$ $P < 0.0001$	$r_s = -0.18$ $P = 0.20$	$r_s = 0.48$ $P < 0.0001$	$r_s = 0.04$ $P = 0.75$	$r_s = -0.03$ $P = 0.78$	$r_s = -0.11$ $P = 0.38$
Number of objects visited			$r_s = 0.23$ $P = 0.07$	$r_s = -0.89$ $P < 0.0001$	$r_s = -0.10$ $P = 0.41$	$r_s = 0.09$ $P = 0.41$	$r_s = 0.13$ $P = 0.38$
Number of squares visited				$r_s = -0.16$ $P = 0.25$	$r_s = -0.12$ $P = 0.38$	$r_s = 0.09$ $P = 0.41$	$r_s = 0.20$ $P = 0.12$
Time to 1st object					$r_s = 0.11$ $P = 0.38$	$r_s = -0.13$ $P = 0.38$	$r_s = -0.11$ $P = 0.38$
Latency to leave holding area						$r_s = -0.11$ $P = 0.38$	$r_s = -0.27$ $P = 0.03$
Scented bedding approach rate							$r_s = 0.06$ $P = 0.60$

Spearman (r_s) correlational analyses were used. P -values were adjusted for multiple comparison using false discovery rates (FDR). Results considered statistically significant are shown in bold.

the body directly (i.e., plasma carotenoid levels) is a more appropriate assessment of carotenoid resources that putatively affect physiological systems and thus behavior.

Thus models included the following main predictors: plasma carotenoid concentration, species and sex. We also included the covariates body mass, Julian date, time of day and temperature and the factors cloud cover and experimental group (i.e., dietary treatment to control for group effects); running models without the group variable returned qualitatively similar results in our main analyses (data not shown). Finally, in order to determine whether the influence of circulating carotenoid levels on behaviors differed between the species or sexes, we also included the following interactions in the models: carotenoid level \times species and carotenoid level \times sex, and we included the interaction between carotenoid level \times group to test whether the influence of carotenoid levels on behavioral responses differed between experimental groups. Following the recommendation of Zuur et al. (2009), all non-significant interaction terms were removed from the models. We summarized parameter effects using a Type III (simultaneous) sum of squares in our main analysis.

Finally, we used Spearman's rank correlation test to determine whether exploratory behavior and activity level PCs were correlated, and Wilcoxon rank sum tests to determine whether individuals approached and interacted with the scented and unscented bedding equally. Specifically, separately for each species, we compared the rate at which individuals approached the scented versus unscented bedding and the amount of time individuals spent

interacting with the scented versus unscented bedding. All analyses were performed with R (v.3.0.2; R Core Team 2013). Modeling assumptions (i.e., heterogeneity of variance, normality of residuals) were validated through visual inspection of residual plots following Zuur et al. (2009). Arcsine transformations were applied to all proportion data; when necessary, other variables were ln-transformed to improve model fit.

3. Results

3.1. Circulating carotenoid levels and body mass

Prior to carotenoid supplementation, and at the end of the depletion period, all birds had very low levels of circulating carotenoids (mean \pm SE; mallards: $2.1 \pm 0.19 \mu\text{g ml}^{-1}$; pintails: $0.9 \pm 0.05 \mu\text{g ml}^{-1}$). Importantly, at this stage, circulating carotenoids levels did not differ among treatment groups in either species (mallards: $F_{5,43} = 0.55$, $P = 0.74$; pintails: $F_{5,45} = 0.10$, $P = 0.99$). Similarly, body mass did not differ among treatment groups in either species (mallards: $F_{5,43} = 1.03$, $P = 0.41$; pintails: $F_{5,45} = 0.07$, $P = 0.99$). However, for both species, 13 weeks of dietary carotenoid supplementation strongly affected levels of plasma carotenoids (mallards: $F_{5,42} = 56.70$, $P < 0.0001$; pintails: $F_{5,44} = 54.55$, $P < 0.0001$). For mallards, there was also a significant effect of sex on plasma carotenoid concentrations ($F_{1,42} = 10.99$, $P = 0.002$): males generally had higher concentrations of carotenoids in circulation relative to females. In contrast, there was no significant

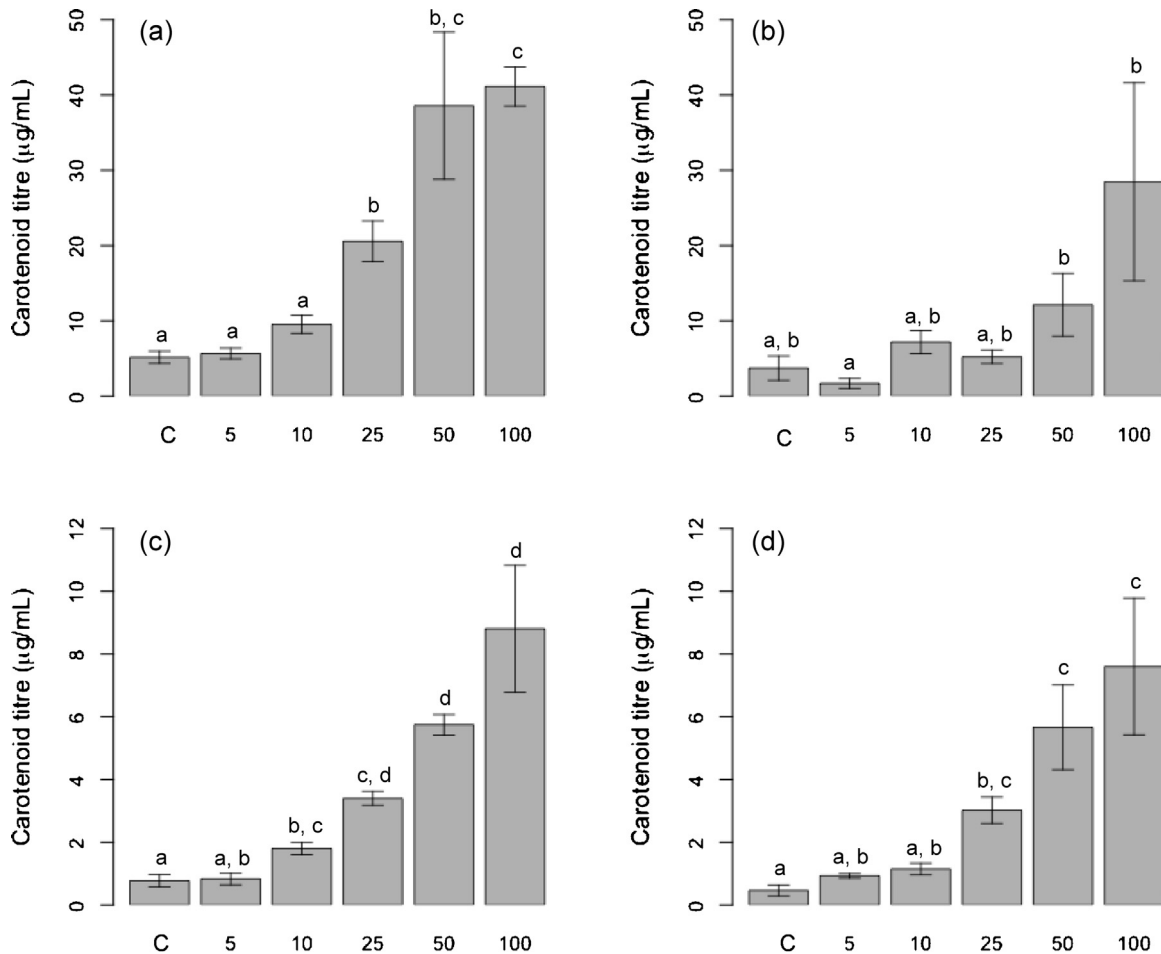


Fig. 2. Effect of diet treatment on plasma carotenoid concentrations at the end of the experiment in (a) male mallards, (b) female mallards, (c) male pintails and (d) female pintails. Shown are means and standard error. Bars marked with different letters differ significantly from one another (posthoc pairwise comparisons, $P < 0.05$ [Tukey's adjustment for multiple comparisons]).

effect of sex on plasma carotenoid levels in pintails ($F_{1,44} = 1.84$, $P = 0.18$). At the time of the behavioral trials, dietary carotenoid treatment was still a significant predictor of plasma carotenoid levels in both species (mallards: $F_{5,42} = 19.09$, $P < 0.0001$; pintails: $F_{5,44} = 41.80$, $P < 0.0001$) and, as before, sex significantly affected plasma carotenoid levels in mallards ($F_{1,42} = 32.27$, $P < 0.0001$) but not pintails ($F_{1,44} = 2.45$, $P = 0.12$). However, pairwise comparisons showed that variation in plasma carotenoid levels did not reflect the underlying dietary carotenoid doses; i.e., circulating carotenoid levels frequently did not differ between dietary treatment groups (Fig. 2).

3.2. Principal components analysis

From the principal components analysis of exploratory behavior variables, we retained two axes that explained a total of 54.7% of the variation in exploratory behavior (Table 2a). The first principal component (exploration PC1) loaded negatively with time to approach a first object and time to approach a second object and positively with both object visitation rate and time spent interacting with novel objects. We therefore interpret higher PC1 scores as indicating birds that were more exploratory (i.e., quicker to approach novel objects and interacting with novel objects more frequently and for longer periods). The second principal component (exploration PC2) loaded negatively with latency to leave the holding area and positively with both the number of approaches to, and time spent in, the scented bedding. Thus we interpret higher PC2 scores as indicating birds that were more exploratory (i.e., were quicker to leave the holding area and interacted more with the scented bedding).

Principal components analysis of activity level produced a single axis explaining 93.9% of the total variation (Table 2b). This principal component (activity level PC1) loaded positively with both the amount of time spent active (e.g., flying, running, and walking) and the number of transitions between different arena sections. We therefore interpret higher PC1 scores as indicating birds that were more active during behavioral trials. Finally, activity level was not correlated with exploration PC2 ($r_s = 0.11$, $P = 0.26$), but was strongly and positively correlated with exploration PC1 ($r_s = 0.54$, $P < 0.0001$).

3.3. Exploratory behavior

Exploration PC1 did not vary in relation to plasma carotenoid concentrations ($F_{1,85} = 0.04$, $P = 0.85$). Similarly, there was no significant effects of species ($F_{1,85} = 1.65$, $P = 0.20$), sex ($F_{1,85} = 0.02$, $P = 0.90$), body mass ($F_{1,85} = 0.84$, $P = 0.36$), date ($F_{1,85} = 0.22$, $P = 0.64$), time of day ($F_{1,85} = 2.90$, $P = 0.09$), temperature ($F_{1,85} = 1.03$, $P = 0.31$), cloud cover ($F_{2,85} = 1.09$, $P = 0.34$) or experimental group ($F_{5,85} = 0.66$, $P = 0.66$) on exploration PC1.

In contrast, circulating carotenoid levels were significantly associated with exploration PC2 scores ($F_{1,85} = 8.09$, $P = 0.0056$). More specifically, individuals with higher concentrations of circulating carotenoids exhibited greater PC2 scores (i.e., were more exploratory; Fig. 3). To ensure that our result was not driven by a relatively small number of individuals with very high plasma carotenoid levels, we repeated this analysis after removing birds with the most extreme plasma carotenoid concentrations (i.e., $>50 \mu\text{g ml}^{-1}$, $n = 2$). The results of this analysis were consistent with that on the full dataset, i.e., exploration PC2 was significantly and positively associated with circulating carotenoid levels ($F_{1,83} = 7.09$, $P = 0.009$). Furthermore, when we applied a strict exclusion criteria to our dataset (i.e., exclude all birds with carotenoid levels exceeding $25 \mu\text{g ml}^{-1}$, $n = 11$), we again found a positive and significant correlation between carotenoid level in circulation and exploration PC2 ($F_{1,74} = 4.07$, $P = 0.04$). There was, however, no significant effect of species ($F_{1,85} = 2.20$, $P = 0.14$), sex ($F_{1,85} = 1.10$, $P = 0.30$), body

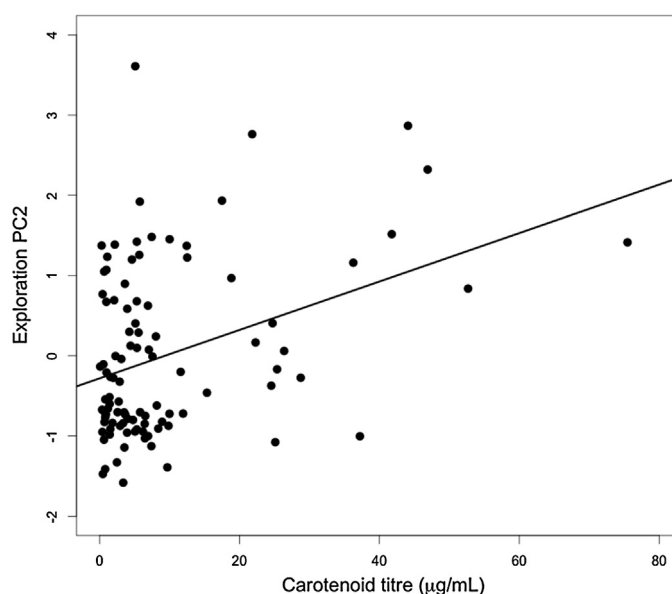


Fig. 3. Relationship between circulating carotenoid levels ($\mu\text{g ml}^{-1}$) and exploration PC2. Higher exploration PC2 values represent individuals that are more exploratory (i.e., individuals that leave the holding area and enter the test arena more quickly and approached and entered the predator-scented bedding more frequently).

mass ($F_{1,85} = 1.70$, $P = 0.20$), date ($F_{1,85} = 0.006$, $P = 0.94$), time of day ($F_{1,85} = 0.04$, $P = 0.85$), temperature ($F_{1,85} = 0.65$, $P = 0.42$), cloud cover ($F_{2,85} = 1.12$, $P = 0.33$) or experimental group ($F_{5,85} = 1.29$, $P = 0.28$) on exploration PC2.

3.4. Activity level

Activity level PC1 was not related to circulating carotenoid concentration ($F_{1,85} = 0.02$, $P = 0.90$). Similarly, activity level did not differ significantly by sex ($F_{1,85} = 1.57$, $P = 0.21$), body mass ($F_{1,85} = 3.54$, $P = 0.06$), date ($F_{1,85} = 1.77$, $P = 0.19$), time of day ($F_{1,85} = 3.92$, $P = 0.051$), temperature ($F_{1,85} = 0.08$, $P = 0.77$), cloud cover ($F_{2,85} = 0.23$, $P = 0.79$) or experimental group ($F_{5,85} = 0.52$, $P = 0.76$). Finally, there was a marginal, but non-significant effect of species ($F_{1,85} = 3.32$, $P = 0.07$), with pintails tending to be less active compared to mallards.

3.5. Scented versus unscented bedding

For both species, there was no significant difference in either the rate at which the birds approached the scented versus the unscented bedding (mallard: $W = 1204$, $P = 0.98$; pintail: $W = 1341.5$, $P = 0.65$) or the time they spent interacting with the scented versus the unscented bedding (mallard: $W = 1086.5$, $P = 0.41$; pintail: $W = 1185$, $P = 0.39$).

4. Discussion

In two species of *Anas* waterfowl, we tested whether levels of circulating carotenoids influenced two common personality traits (i.e., exploratory behavior and activity level) by placing birds in a novel-environment incorporating novel objects and a potential predator threat (fox urine scent). We found that circulating carotenoid levels were positively associated with some measures of exploratory behavior (exploration PC2): birds with higher levels of circulating carotenoids entered the test arena sooner and approached and entered predator-scented bedding material more frequently relative to birds with low concentrations of circulating carotenoids. We also found an association between exploratory

behavior (exploration PC1) and activity level, which is consistent with previous studies showing phenotypic and genetic correlations frequently occur among personality traits (e.g., van Oers et al., 2004a,b), including exploration and activity (Wilson and Godin, 2010).

Interestingly, in both species, individuals did not appear to discriminate between the scented and unscented bedding (i.e., there was no difference in either the approach rate or the time spent interacting with scented versus unscented bedding trays). These findings suggest that birds either failed to detect the scent or that the presence of predator chemical cues does not induce avoidance behaviors in waterfowl. Learned recognition of predator specific characteristics is observed in a range of species, including birds (e.g., Sonerud, 1985). Thus, the lack of response to red fox scent may result from our birds not having learned to associate red fox scent with a predation event such as nest loss. Unfortunately, we have no way of testing between these two explanations at this time, but we suggest that interactions with the scented bedding represent general exploratory behavior of a novel object and not a potential predator threat induced response.

We surmise that carotenoids may promote aspects of exploratory behavior either by providing antioxidant protection to tissues in the body that are relevant to navigation and locomotion or by boosting systematic health and immune function. In the first instance, carotenoids might be expected to influence physiological performance at the behavioral level if they provide antioxidant protection to muscles (Ji, 1999; Powers et al., 2004) or other body tissues relevant to animal movement (e.g., eyes, brain). For example, physical activity can lead to increases in reactive oxygen species (ROS) production (O'Neill et al., 1996; Larcombe et al., 2008), which can in turn disrupt the reduction–oxidation balance in muscle tissue and cause oxidative damage and muscle fatigue (Powers et al., 2004) with negative consequences for mobility and perhaps also for the likelihood of exploration. In European greenfinches, Herborn et al. (2011) found that fast-exploring individuals (i.e., those with a reduced latency to explore novel objects) had a greater capacity to resist oxidation by the pro-oxidant, hypochlorous acid (HOCl). In our study, ducks that were quick to enter the novel environment test arena had higher levels of circulating carotenoids. Taken together, these findings suggest that increased carotenoid resources may enhance an individual's ability to resist oxidative stress with consequences for exploratory behavior in birds.

In the second instance, resource-acruing personality traits, such as exploration and activity, appear to be related to an individual's past or current immune status (Butler et al., 2012; Grindstaff et al., 2012; Männiste et al., 2013), and increases in carotenoid resources are associated with enhanced immunocompetence and health of individuals in a range of species (e.g., Blount et al., 2003; McGraw and Ardia, 2003), including mallards (Butler and McGraw, 2012). Thus, a putative explanation for our results is that birds with high levels of circulating carotenoids had increased antioxidant protection of relevant body tissues or enhanced immunocompetence/general health. The fact that carotenoid resources influenced one metric of exploration but not our other measure of exploration or general activity may simply be a result of the orthogonal nature of PC axes, or it may imply that, at least in mallards and pintails, exploratory behaviors and activity may not be governed by a common underlying physiological mechanism.

In the currently study, we experimentally elevated circulating carotenoid levels via dietary carotenoid supplementation using a range of carotenoid doses (i.e., 5, 10, 25, 50 or 100 $\mu\text{g g}^{-1}$ of xanthophyll carotenoids). We chose a series of doses of increasing concentrations to explore the potential dose-dependent effects of carotenoid resources on exploratory behavior. We found, however, that while dietary carotenoid intake did influence circulating carotenoid levels, these changes were more graduated than expected

and did not specifically reflect carotenoid levels in the diet of individuals, which is likely due to intra-individual variation in carotenoid assimilation ability. Uncoupling of dietary intake and plasma carotenoid circulation has also been reported in the zebra finch (*Taeniopygia guttata* [McGraw et al., 2003]). Thus, while dietary access appears to elevate carotenoid levels in circulation, the degree to which carotenoid intake contributes to plasma carotenoid levels requires further study, and we suggest caution should be used when inferring carotenoid resources from dietary levels alone. Similarly, we suggest that circulating carotenoid levels may not always provide a clear representation of dietary access to carotenoids. Importantly, the lack of association between diet and plasma carotenoids in our study compelled us to examine the effect of carotenoid resources in the current study directly (i.e., via circulating carotenoids levels), which we suggest is a more accurate measure of carotenoid availability in this instance and thus provides stronger evidence for an effect of carotenoid resources on exploratory behavior than that which could be inferred from an effect of dietary carotenoid levels.

An important issue to consider is whether the high plasma carotenoid levels observed in the current study are biologically meaningful. We observed carotenoid levels as high as 75 $\mu\text{g ml}^{-1}$, (mean \pm SD; $9.4 \pm 12.9 \mu\text{g ml}^{-1}$), though the majority of our data (89%) fell below 25 $\mu\text{g ml}^{-1}$. While there is no available data for wild adults of either species, average circulating carotenoid levels in mallard ducklings exceed 15 $\mu\text{g ml}^{-1}$ (Butler and McGraw, 2010) and carotenoid concentrations in wild birds range from 0.5 to 75 $\mu\text{g ml}^{-1}$ (McGraw, 2006), suggesting the values observed in our study can be cautiously considered as biologically relevant. Moreover, given that we found the same positive association between circulating carotenoid levels and exploratory behavior (i.e., exploration PC2) when analysis was restricted to individuals with carotenoid levels less than 25 $\mu\text{g ml}^{-1}$, we suggest the link between carotenoid availability and aspects of exploratory behavior observed in this study is also biologically relevant and meaningful.

More generally, our results can offer insight into carotenoid physiology and accumulation in waterfowl. First, we observed that male mallards exhibited the highest levels of circulating carotenoids in our study, which supports the notion that birds displaying carotenoid-dependent ornamentation possess physiological adaptations that enable them to absorb or accumulate greater amounts of carotenoids (McGraw, 2005). Next, it has been suggested that there may be a physiological ceiling to carotenoid absorption, even in the face of strong selection to accumulate as many carotenoids as possible for health and coloration purposes (McGraw, 2006). We found no difference in circulating carotenoid levels between birds fed 50 or 100 $\mu\text{g g}^{-1}$ of carotenoids, despite the doubling of the dietary dosage. Though it is uncertain whether a dose of 100 $\mu\text{g g}^{-1}$ of carotenoids reflects the natural food sources of waterfowl, the fact that the increased dosage did not translate into higher plasma carotenoid levels suggests xanthophyll carotenoid saturation may occur at a dose of approximately 50 $\mu\text{g g}^{-1}$ in these species.

In summary, our results suggest that circulating carotenoid levels are correlated with the expression of some aspects of a common personality trait (i.e., exploratory behavior) in adult male and female mallards and northern pintails. The fact that carotenoid availability did not differentially affect behavior of the two species (only one of which is carotenoid-colored) suggests that, at least as it relates to carotenoid pools that may enhance exploration and activity, devotion of pigments to the integument does not alter endogenous carotenoid allocation trade-offs or strategies. Finally, though the mechanisms underlying the relationship between carotenoid availability and exploratory behavior are unclear, our findings demonstrate a link between carotenoid status and the

expression of an individual's personality. These results highlight the potential for specific nutrients to contribute to inter-individual variation in personality, and we suggest that future studies investigating the links between carotenoid nutrition, physiology and personality will help deepen our understanding of the proximate mechanisms driving variation in personality traits.

Acknowledgements

We thank the staff of the Department of Animal Care Technologies at Arizona State University for caring for our animals, Alison Ossip-Klein and Erin Nelson for mixing supplementary diets, and Mike Butler, Alison Ossip-Klein and Mathew Toomey for their advice and help with logistics involved in conducting this study. This manuscript was greatly improved by comments from Andy Sih Terje Laskemoen, Becky Cramer and an anonymous reviewer. This research was funded by the National Science Foundation (grant numbers NSF/IOS-0746364 and –0925633 to KJM) and the Barrett Honors College of Arizona State University (to KLP).

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