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RESEARCH ARTICLE

Eat yourself sexy: how selective macronutrient intake influences the expression of a visual signal in common mynas

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ABSTRACT

Producing colored signals often requires consuming dietary carotenoid pigments. Evidence that food deprivation can reduce coloration, however, raises the question of whether other dietary nutrients contribute to signal coloration, and furthermore, whether individuals can voluntarily select food combinations to achieve optimal coloration. We created a two-way factorial design to manipulate macronutrient and carotenoid access in common mynas (*Acridotheres tristis*) and measured eye patch coloration as a function of the food combinations individuals selected. Mynas had access to either water or carotenoid-supplemented water and could either eat a standard captive diet or choose freely between three nutritionally defined pellets (protein, lipid or carbohydrate). Mynas supplemented with both carotenoids and macronutrient pellets had higher color scores than control birds. Male coloration tended to respond more to nutritional manipulation than females, with color scores improving in macronutrient- and carotenoid-supplemented individuals compared with controls. All mynas consuming carotenoids had higher levels of plasma carotenoids, but only males showed a significant increase by the end of the experiment. Dietary carotenoids and macronutrient intake consumed in combination tended to increase plasma carotenoid concentrations the most. These results demonstrate for the first time that consuming specific combinations of macronutrients along with carotenoids contributes to optimizing a colorful signal, and point to sex-specific nutritional strategies. Our findings improve our knowledge of how diet choices affect signal expression and, by extension, how nutritionally impoverished diets, such as those consumed by birds in cities, might affect sexual selection processes and, ultimately, population dynamics.

KEY WORDS: *Acridotheres tristis*, Carotenoids, Coloration, Foraging, Macronutrients, Signaling

INTRODUCTION

For animals, daily search and acquisition of food is essential in order to meet their energy and nutritional requirements (Robbins, 1993). Foods are complex blends of many nutrients, each one of which has its own unique or multiple physiological functions (Simpson and Raubenheimer, 2012). Macronutrients are 'essential' nutrients and are required in large amounts. They include lipids and carbohydrates, which play a central role in metabolism and energy storage, and proteins, which are fundamental to animals' growth and reproduction (McWilliams, 2011). Micronutrients are required in smaller quantities and include molecules such as carotenoids, which animals use to produce colorful signals (Blount and McGraw, 2008). Nutritional and energy requirements can vary across categories of individuals (e.g. males versus females) and/or across an individual's lifespan (e.g. as a function of age, migratory status) (Simpson et al., 2010). Therefore, a successful forager could not only sample available foods for their energy content, but also to ingest a specific balance of nutrients that meets physiological needs (Simpson and Raubenheimer, 2012).

The production of colored signals involves specific energetic and nutritional inputs. Carotenoid-based signals rely heavily upon dietary access to carotenoid micronutrients (Peneaux et al., 2021). Carotenoids are biologically active pigments synthetized *de novo* by plants, but most vertebrates can only acquire them through their diet (Goodwin, 1984; Isaksson, 2009; Latscha, 1990). As a signal often used by conspecifics to select suitable mates or ward off rivals (Andersson and Simmons, 2006; Blount and McGraw, 2008), individuals should actively seek nutrients that benefit signal expression. Therefore, individuals should have the ability to find, recognize and consume food items that enhance signal expression, and should prioritize doing so at times of signal production. In support of this idea, captive and field studies in birds that display carotenoid-based structures have shown that individuals, typically males, select food items that are enriched in carotenoid pigments (Senar et al., 2010; Walker et al., 2014).

Studies on the importance of diet and nutrition on color production have focused on pigment availability and potential limitations of carotenoids (Alonso-Álvarez et al., 2004; Hill, 2006; Hill et al., 2009; McGraw et al., 2004; Peneaux et al., 2021). However, carotenoid-based coloration and metabolism are also likely influenced by the uptake of other essential nutrients (see Peneaux et al., 2021 for a review). Dietary fats, for example, contribute to carotenoid pigment absorption and transport via lipoproteins (Parker, 1996; Toomey et al., 2017; Tyczkowski et al., 1989). Several studies on food deprivation and cholesterol have shown the potential impact of manipulating lipid levels on signal coloration in birds (Hill, 2000; Hill et al., 2009; McGraw and Parker, 2006; McGraw et al., 2005; Pérez-Rodríguez and Viñuela, 2008). Proteins are also necessary to the production of colored signals because they play a structural role by producing keratin, the main constituent of feather barbs, or by forming the array of collagen fibers upon which pigments will be deposited in skin tissues (Murphy and Taruscio, 1995; Shawkey and Hill, 2005). Furthermore, the production of carotenoid coloration could be costly to individuals (Hill, 2000, 2002; McGraw et al., 2005; Shawkey et al., 2006), potentially generating energetic constraints and a reliance on carbohydrates as a rich source of energy. To broaden our understanding of the factors that determine signal expression and quality, we need to quantify jointly the effects of

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carotenoid and macronutrient intake and examine whether individuals can select foods that optimize this interaction.

We tested the hypotheses that dietary carotenoid and macronutrients should contribute to the quality of a carotenoid signal, and that animals have the ability to detect and consume foods containing nutrients that enhance carotenoid signal quality. The common (Indian) myna (*Acridotheres tristis*) is a passerine that displays colored skin features and is known for its ability to select foods of differential macronutrient content (Machovsky-Capuska et al., 2016a; Peneaux et al., 2017, 2020). These features make it an ideal model species to test our hypotheses. We allocated individual mynas to treatments with differential access to dietary carotenoids and macronutrients. We then measured differential change in eye patch coloration both across time and treatments, and as a function of individual dietary consumption. Considering that a high-quality diet is obtained when individuals can select a specific balance of nutrients to maximize their fitness (Raubenheimer et al., 2012), we created a flexible design with nutritionally defined foods for individuals to self-select their macronutrient intake within the proposed treatments.

Our general prediction was that, given the choice, mynas would consume foods in relative proportions that would be associated with more colorful eye patches. More specifically, we also predicted that relatively high lipid intake would be associated with higher plasma carotenoid levels and enhanced coloration. This prediction was based on published evidence that lipids facilitate dietary carotenoid uptake. There are reasons to predict that carbohydrate and protein intake might also be positively associated with enhanced coloration. Indeed, production of carotenoid coloration might be energetically demanding (Hill, 2000, 2002; McGraw et al., 2005; Shawkey et al., 2006) and protein is the main constituent of the microstructure of the myna's colored eye patch (Peneaux et al., 2020). However, increasing consumption of one macronutrient must be offset against reductions in consumption of the others because individuals cannot increase the amount of food they eat indefinitely. We reasoned that the yellow coloration of the myna eye patch is unlikely to be produced by bioconversion of dietary carotenoids and that proteins do not interact directly with carotenoid metabolism. Consequently, we predicted that, in comparison with lipids, the proportion of carbohydrate and protein ingested would be lower in individuals with enhanced eye patch coloration.

MATERIALS AND METHODS

Study species

The common myna, *Acridotheres tristis* (Linnaeus 1766; also proposed to be reclassified as *Sturnus tristis* Christidis and Boles 2008; hereafter myna), is a medium-sized passerine closely related to starlings and one of the most common introduced species globally, including Australia (Grarock et al., 2013; Sol et al., 2011). In Australia, mynas are well established in major urban centers along the east coast (Lowe et al., 2011; Sol et al., 2012a). They are monomorphic, with both males and females displaying yellow to orange colored skin around their eyes and on their beak and legs. Eye patch coloration is known to progressively fade in captive mynas fed a diet of dog pellets (a diet that apparently does not contain dietary carotenoid precursors usable by birds; C. Peneaux, unpublished data; Table S1), despite dog pellets being a preferred food of mynas under natural conditions (Tidemann, 2009). Anatomical examination of eye patch skin tissue has shown that the coloration is produced by the inclusion of carotenoid pigments into the epidermis and the presence of nanostructured collagen in the dermis, but the identity of the carotenoids in the epidermis remains unknown (Peneaux et al., 2020). Mynas are a secondary-cavity nester and typically breed during the warmer months (September to April) (Counsilman, 1974). They are behaviorally monogamous, forming life-long bonds with their partners (Counsilman, 1974), and it is reasonable to assume that this carotenoid-based skin display functions as an indicator of individual quality (Endler, 1980; Svensson and Wong, 2011; Weaver et al., 2018). They are also generalist omnivores and previous work has demonstrated that mynas can select foods based on the food's nutritional content and on their own physiological needs (Machovsky-Capuska et al., 2016a; Peneaux et al., 2017).

Subjects and husbandry

We captured a total of 40 adult birds in Newcastle (NSW, Australia) using a walk-in baited trap (Tidemann, 2009) during the non-breeding season (from 9 June to 21 July 2018). Upon arrival to our captive facilities, mynas were kept in a large outdoor flight aviary (2.0×1.0×2.0 m, height×depth×width) where water and dog pellets (SUPERCOAT® Puppy with Real Meat, 32.4% protein, 15.6% lipid, 52% carbohydrate; Table S1), a classic bait and captive diet for myna (Tidemann, 2009), were provided *ad libitum*. Following the nutritional levels provided by the manufacturer, to the best of our knowledge, dog pellets do not contain dietary carotenoids that mynas could absorb. We maintained the subjects under these conditions (at least 8 weeks) until coloration faded (see 'Coloration measurements' for methods). One week prior to the experiments, we acclimated the birds to individual indoor cages (0.7×0.5×0.8 m) with *ad libitum* water and dog pellets. We tested two successive cohorts of 20 birds owing to space constraints and logistical challenges to measure the daily intake of more individuals simultaneously. We ran experiments from 27 August to 18 November 2018 as maximum signal production was expected to occur at the beginning of the breeding season. During testing, birds were held in indoor individual cages equipped with a perch and a nest box, and spatially arranged such that birds were in close visual and acoustic contact. This arrangement facilitates mynas' adjustment to individual housing (Griffin and Boyce, 2009; Griffin and Haythorpe, 2011; Griffin et al., 2013; Sol et al., 2012b).

All husbandry and experimental procedures were approved by the Animal Care & Ethics Committee of the University of Newcastle (Animal Research Authorities A-2017-718).

Experimental procedure

Fading protocol and treatment groups

Nutritional manipulation started once signal coloration had significantly decreased from the time of capture. Allowing eye patch color to first fade allowed us to standardize eye patch coloration across birds, creating a starting point from which we could track whether and how coloration changed as a function of nutritional intake. Subjects were held in flight aviaries for a minimum of 8 weeks, during which each bird's eye patch coloration was quantitatively measured and blood was collected (see 'Coloration measurements', below) after 2, 4 and 8 weeks in captivity and compared with values at capture. Compared with the time of capture, decreases in coloration and plasma concentration in carotenoids were observed in captured birds after 8 weeks in captivity (see Figs S1 and S2). Throughout the fading phase, coloration and level of plasma carotenoids stayed within the range found under natural conditions in populations of mynas and individuals' body mass increased, confirming that our experimental manipulation did not induce biologically unnatural or unhealthy conditions in our subjects (C. Peneaux, unpublished data; Fig. S2).

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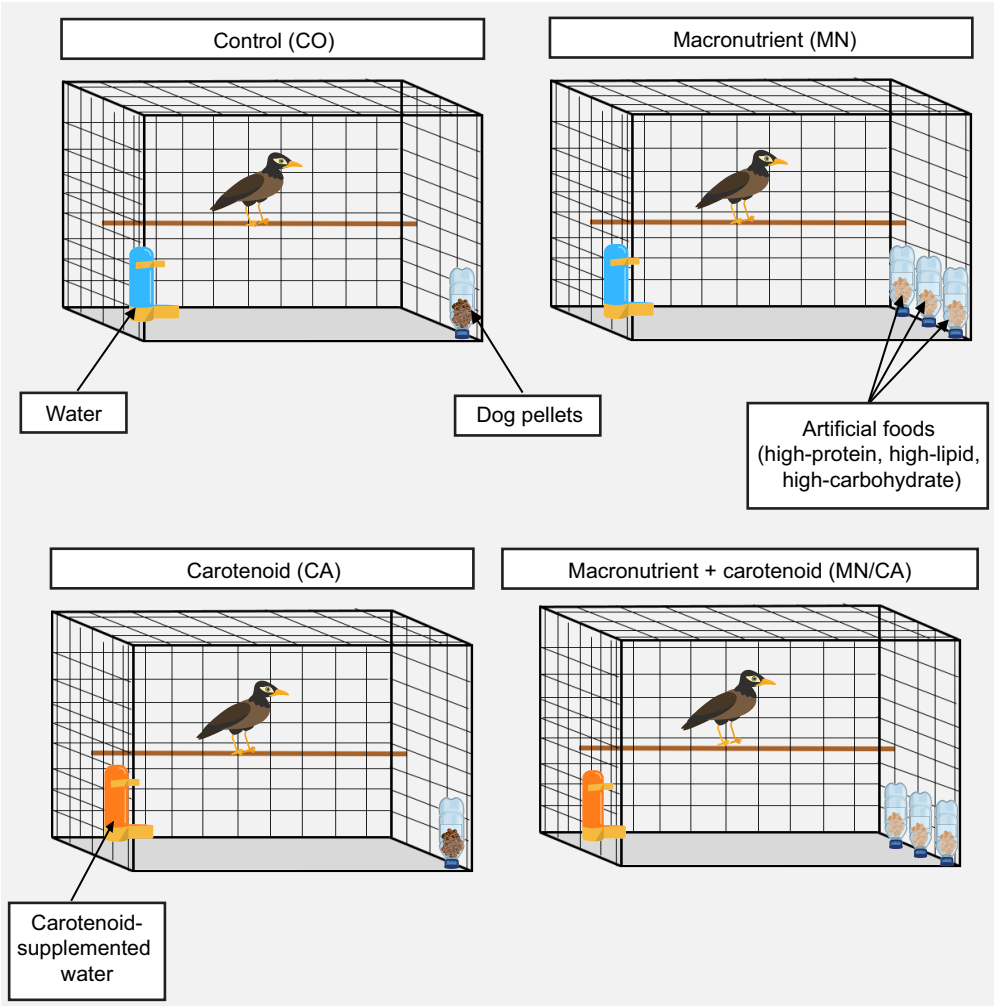

Fig. 1. Schematic of the experimental design. Four treatment groups with different nutritional conditions were created: (1) control group (CO), birds had access to the usual dog pellet diet and drinking water; (2) macronutrient group (MN), birds were allowed a free choice between the three artificial foods (high-protein, high-lipid and high-carbohydrate, each type provided in a separated feeder) and drinking water; (3) carotenoid group (CA), birds were provided with dog pellets and carotenoid-supplemented drinking water; and (4) macronutrient+carotenoid group (MN/CA), birds were given both the three artificial foods and carotenoid-supplemented drinking water. Drinking solutions were provided in a typical birdcage drinker and food pellets were provided in a homemade feeder made of an upside-down 250 ml plastic water bottle with an entry hole at the front, which allowed birds to fit their heads inside.

Male (M) and female (F) test subjects (N=10) were distributed into four treatment groups (Fig. 1): (1) control (CO): dog pellet+water (3 F, 7 M); (2) macronutrient-supplemented (MN): high-protein, high-lipid, high-carbohydrate pellets+water (4 F, 6 M); (3) carotenoid-supplemented (CA): dog pellet+carotenoid-supplemented water (5 F, 5 M); or (4) both macronutrient- and carotenoid-supplemented (MN/CA): high-protein, high-lipid, high-carbohydrate pellets+carotenoid-supplemented water (5 F, 5 M). Subjects were assigned to treatment groups according to their color scores (method described below) measured after fading occurred to establish a coloration range within each group.

Feeding choice

Mynas in the MN and MN/CA groups were presented with a simultaneous choice of three semi-synthetic pellet foods with different macronutrient compositions. Each of these foods was enriched with either proteins (P), lipids (L) or carbohydrates (C). Following Machovsky-Capuska et al. (2016a), the semi-synthetic pellets used in our experimental design were made of ingredients that reflect the nutrient sources (animal protein, human discards, arable crops) that are typically available to mynas in the wild (Table S2). The foods were formulated to be isoenergetic (2600 kcal kg^{-1}) and were balanced for all macrominerals and microminerals to minimize confounding effects between foods. The macronutrient composition for the three foods were as follows: high-protein (79.6% P, 3.3% L, 17.1% C), high-lipid (16.7% P, 76% L, 7.3% C) and high-carbohydrate (7.9% P, 0.1% L, 92% C). All foods were cold pelleted at 60°C (4 mm diameter and 3 mm length, mass range: 0.09–0.11 g). Slight color differences among the pellets allowed the return of spilt food to the correct containers. A total of 25 g of each food was placed in one of three individual plastic bottles (250 ml) attached to the inside of the cages with an opening large enough for birds to reach pellets (Fig. 1). Remaining food pellets were collected every 24 h (between 07:00 and 08:00 h) and weighed to quantify daily macronutrient consumption [difference in mass (g)] of study subjects.

Carotenoid supplementation

Following a study by Baeta et al. (2008), we added a solution of dietary carotenoids lutein and zeaxanthin (available in liquid form, OroGLO® 16 Liquid, Kemin Industries) to the drinking water of CA and MN/CA birds. Although the composition of carotenoid compounds in the diet of wild *A. tristis* is unknown, lutein and zeaxanthin are yellow pigments and some of the most abundant carotenoids found in the foods and blood of many bird species (Goodwin, 1984; Latscha, 1990; McGraw, 2006). They are also often involved in the production of integumental coloration by being directly deposited in the integument or by being converted into new pigments before deposition (e.g. dehydrogenation into other xanthophylls) (Butler and McGraw, 2010; García-de Blas et al., 2011, 2016; McGraw et al., 2006; Weaver et al., 2018). Although HPLC analyses by our group have confirmed the presence

of lutein – but also β-cryptoxanthin (a red pigment) – in the plasma of mynas, the study was not designed to detect zeaxanthin and did not analyze myna skin because the samples were processed within the context of human research on carotenoids (Peneaux et al., 2020). Reasoning that β-cryptoxanthin is involved in the production of red-converted pigments via hydroxylation ketolation (Weaver et al., 2018) and that color produced in mynas is predominantly yellow, we selected lutein and zeaxanthin as candidates for supplementation.

The maximum dose provided in this experiment (160 µg ml^{-1}) is included in the range generally used for supplementation in previous studies on carotenoid supplements in birds (Baeta et al., 2008; Blount et al., 2003; Hill, 1992; McGraw et al., 2001; Navara and Hill, 2003). Moreover, the levels of plasma carotenoids in supplemented birds during this experiment (mean: 2.14 µg ml^{-1}; min.: 0.09 µg ml^{-1}; max.: 5.82 µg ml^{-1}) stayed within natural range in this species (mean: 0.92 µg ml^{-1}; min.: 0.04 µg ml^{-1}; max.: 7.63 µg ml^{-1}; C. Peneaux, unpublished data), confirming that the carotenoid dose did not result in unnatural levels of plasma carotenoids. The diluted solution was the sole drinking opportunity for the carotenoid-supplemented birds and was provided in an opaque drinking container to protect the pigments from light degradation and to avoid neophobic responses to the orange tint of the water. Birds were progressively introduced to the carotenoid solution by slowly increasing its concentration in the drinking water, until reaching the desired concentration by the end of the second week of experimentation. Similar to food consumption, liquid consumption was measured every 24 h and daily carotenoid intake (mg) was calculated.

Coloration measurements

Inter-individual differences in the color of the eye-patch was determined from photographs taken weekly during the experiment. The photos were taken by placing the bird inside a custom-designed enclosure that provided standardized conditions to maintain identical object to lens distance, shutter, exposure and flash settings, as well as standard light conditions across photographs (see Peneaux et al., 2021 for a detailed description of the device and for more information about standardization). The device consisted of a white lidded bucket (30×30×35 cm), painted with several layers of black paint to obscure external light, and with LED lights fixed to the inside of the lid to provide the only source of light inside the apparatus. Birds were introduced inside the device through a curtained opening at the front and placed in a standardized position against a transparent shelf. An opening on the top of the device was made to fit a camera lens to capture photos with both the measured displays and a color standard, which allowed us to standardize coloration, brightness and size on all pictures. Like many other carotenoid-based displays, the reflectance spectra of colored eye patch skin shows a first peak in the UV followed by a larger peak and plateau at longer wavelengths (Peneaux et al., 2020). Several studies have shown that measuring the visible region of the spectrum via digital photography allow the capture of biologically meaningful variation in carotenoid-based coloration (Giraudeau et al., 2012; Pérez-Rodríguez, 2008; Vergara et al., 2015; Villafuerte and Negro, 1998). This method also allowed us to considerably reduce handling time for each bird, compared with the spectrometer method.

Using a Canon EOS 550D with an 18–55 mm lens, we took two photos of the left side of the head for each bird and analyzed them with a custom-made MATLAB program (available from Mendeley Data at doi:10.17632/djpymvcp2m.1; Peneaux et al., 2021). The program allowed us to accurately select the eye-patch area and extract RGB, hue, chroma and brightness values of the selected area. As the program assigns hue angle values between −180 and 180 deg, with red set at −120 deg, lower hue scores correspond to redder birds. Values for the two pictures of each bird were averaged for statistical analyses. Using principal component analysis (PCA) on the averaged hue, chroma and brightness values, we obtained an overall color score (PC1) for each bird. PC1 summarized over 60% of coloration variation and loaded positively on hue (component loading >92%) and brightness (component loading >64%) and negatively on chroma (component loading >73%). This PC1 score is consistent with previous observations of color variation within the eye patch of common mynas (Peneaux et al., 2020), where highly pigmented displays had the lowest hue, lowest brightness and highest chroma and poorly pigmented displays had the highest hue, highest brightness and lowest chroma. To simplify graphical visualization, we multiplied PC1 scores by −1 so that the highest PC1 scores corresponded to the most enhanced coloration outputs.

Plasma analysis

Once every 2 weeks, 150–200 µl of whole blood samples were collected from each bird by pricking the brachial vein. The blood was immediately centrifuged (5000 ***g***, 15 min) to allow collection of the plasma. The plasma was stored in 1.5 ml Eppendorf tubes at −80°C for up to 5 months before analysis. Plasma carotenoid concentration was analyzed using a standardized colorimetric technique (see Alonso-Álvarez et al., 2004). A volume of 20 µl of plasma was diluted in 180 µl of absolute ethanol, mixed using a vortex and centrifuged (1500 ***g***, 10 min) to precipitate the flocculent proteins. The supernatant was analyzed using a spectrophotometer to determine the optical density of the carotenoid peak at 450 nm. Carotenoid concentration was determined from a standard lutein curve.

Statistical analyses

To assess differences in coloration, plasma carotenoid levels and the consumption of food, we used linear mixed models (LMMs) [R packages lme4 (https://CRAN.R-project.org/package=lme4) and lmerTest (https://CRAN.R-project.org/package=lmerTest)] to test for treatment, sex and interaction effects. Random factors in all models were defined as individual identity and cohort. Final *P*-values were obtained using type III *F*-tests with Kenward–Roger approximations for degrees of freedom (R package lmerTest). Color scores (PC1) were used for the analysis. Plasma carotenoid data were log transformed to achieve normality. For both coloration and plasma carotenoid, analyses were run both on the four treatment groups and separately on the CA and MN/CA groups with carotenoid intake as a covariate. These two groups had access to pigments and hence could potentially change color, but only one of them (MN/CA) could vary its nutritional intake. This is therefore a key comparison to understand how nutritional selection impacts signal coloration.

For nutritional analyses, the response was the weekly percentage of food consumed. An additional predictor 'nutrient' was added to the analysis and constituted a three-level categorical variable giving the dietary content of each food (P, L or C). Individual body mass was added as a covariate. *Post hoc* tests (contrasts) were conducted using the emmeans package (https://CRAN.R-project.org/package=emmeans), and *P*-values were adjusted for the contrast between four treatment groups using the Tukey's test (adjusted *P*-values noted as *P*′). We used repeated-measures generalized additive models (GAMs) (mgcv package, https://CRAN.R-project.org/package=mgcv) to determine the interactive effect of macronutrient intake on color scores and plasma carotenoid concentrations. For all GAMs, we analyzed the main effects of P, L and C intake (g) and their interaction, using color scores or plasma carotenoid concentrations as factors. We used thin-plate

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spline procedures to generate 2D response surfaces using the fields package (https://CRAN.R-project.org/package=fields) (Wood, 2017). In each surface, red regions indicate the greatest values for a given response (i.e. highest coloration scores or highest plasma carotenoid concentrations), with these values decreasing as colors change to blue. Contour lines within each surface are isolines indicating equal values for each trait along that line. All analyses were performed in the statistical programming environment R 3.5.3 (https://www.r-project.org/).

RESULTS

Change in coloration and plasma carotenoid levels

Color scores

Eye patch coloration changed as a function of treatment and time when pooling all birds (Table 1, Fig. 2A). Coloration did not differ at day 1 across the four treatment groups (LMM, day 1: $F_{3,36}$=0.79, P=0.51). However, color scores were significantly different by the end of the experiment at day 28 (LMM, day 28: $F_{3,36}$=4.40, P=0.01). *Post hoc* analyses revealed that color scores in the MN/CA group were significantly higher than in both CO (Tukey's pairwise comparisons, P'=0.02) and MN (P'=0.047) groups at the end of the experiment. We found no differences in coloration between the CA group and any of the other groups (P'>0.15). In the carotenoid-supplemented groups, amount of carotenoid ingested had no effect on coloration (Table 1).

A marginally non-significant three-way interaction was detected, involving treatment, time and sex on color scores (Table 1, Fig. S3A). Because treatment and time were key experimental manipulations in the present study, and their effects are of central interest to our questions, we considered it justified to dissect the top-level three-way interaction further to determine whether the effects of these variables might be qualified by sex. Female coloration did not change over the experiment period (LMM, day 1: $F_{3,13}$=2.30, P=0.13; day 28: $F_{3,13}$=1.23, P=0.32) but male coloration did (LMM, day 1: $F_{3,19}$=0.26, P=0.86; day 28: $F_{3,19}$=6.11, P=0.004). At day 28, MN/CA males had significantly higher color scores than CO (P'=0.005) and MN males (P'=0.03).

Plasma carotenoids

There was a marginally non-significant difference in carotenoid levels at day 1 across the four treatment groups (LMM, day 1: $F_{3,36}$=2.49, P=0.08), possibly influenced by sex whereby some cross-treatment variation emerged in females but less so in males (Fig. S3B). There was an interactive effect of treatment and time on plasma carotenoid levels (Table 1, Fig. 2). By the end of the

Table 1. Results of linear mixed models examining the effects of treatment, time, sex and nutrient type on eye patch coloration, plasma carotenoid levels and nutrient intake in common mynas

Response	Predictor	d.f.	*F*	*P*
Color scores (PC1) – all groups	Treatment	3,31	**5.49**	**0.004**
	Time	4,128	1.31	0.27
	Sex	1,31	0.59	0.45
	Treatment×Time	12,128	**2.56**	**0.005**
	Treatment×Sex	3,31	1.39	0.26
	Time×Sex	4,128	1.51	0.2
	Treatment×Time×Sex	12,128	1.73	0.067
Color scores (PC1) – carotenoid-supplemented groups only	Treatment	1,25	2.26	0.15
	Time	1,20	0.15	0.70
	Sex	1,89	0.91	0.34
	Carotenoid intake	1,90	0.01	0.92
	Treatment×Time	1,26	0.16	0.69
	Treatment×Sex	1,78	0.11	0.74
	Time×Sex	1,75	**12.06**	**<0.0001**
	Treatment×Time×Sex	1,75	0.39	0.53
Plasma carotenoid levels – all groups	Treatment	3,31	**41.64**	**<0.0001**
	Time	2,64	**41.23**	**<0.0001**
	Sex	1,32	0.46	0.51
	Treatment×Time	6,64	**16.59**	**<0.0001**
	Treatment×Sex	3,32	0.77	0.52
	Time×Sex	2,64	**4.18**	**0.02**
	Treatment×Time×Sex	6,64	0.89	0.51
Plasma carotenoid levels – carotenoid-supplemented groups only	Treatment	1,49	2.04	0.16
	Time	1,49	1.32	0.26
	Sex	1,45	**5.45**	**0.03**
	Carotenoid intake	1,39	0.71	0.4
	Treatment×Time	1,49	0.01	0.91
	Treatment×Sex	1,38	0.19	0.67
	Time×Sex	1,35	3.39	0.07
	Treatment×Time×Sex	1,35	0.79	0.38
Nutritional intake – CA and MN/CA groups	Treatment	1,14	0.00	1
	Nutrient	2,212	**119.35**	**<0.0001**
	Sex	1,15	0.00	1
	Body mass	1,8	0.00	1
	Treatment×Nutrient	2,212	**43.37**	**<0.0001**
	Treatment×Sex	1,13	0.00	1
	Nutrient×Sex	2,212	**9.75**	**<0.0001**
	Treatment×Nutrient×Sex	2,212	**9.75**	**<0.0001**

P-values for significant factors (α<0.05) are shown in bold and marginal effects are underlined.

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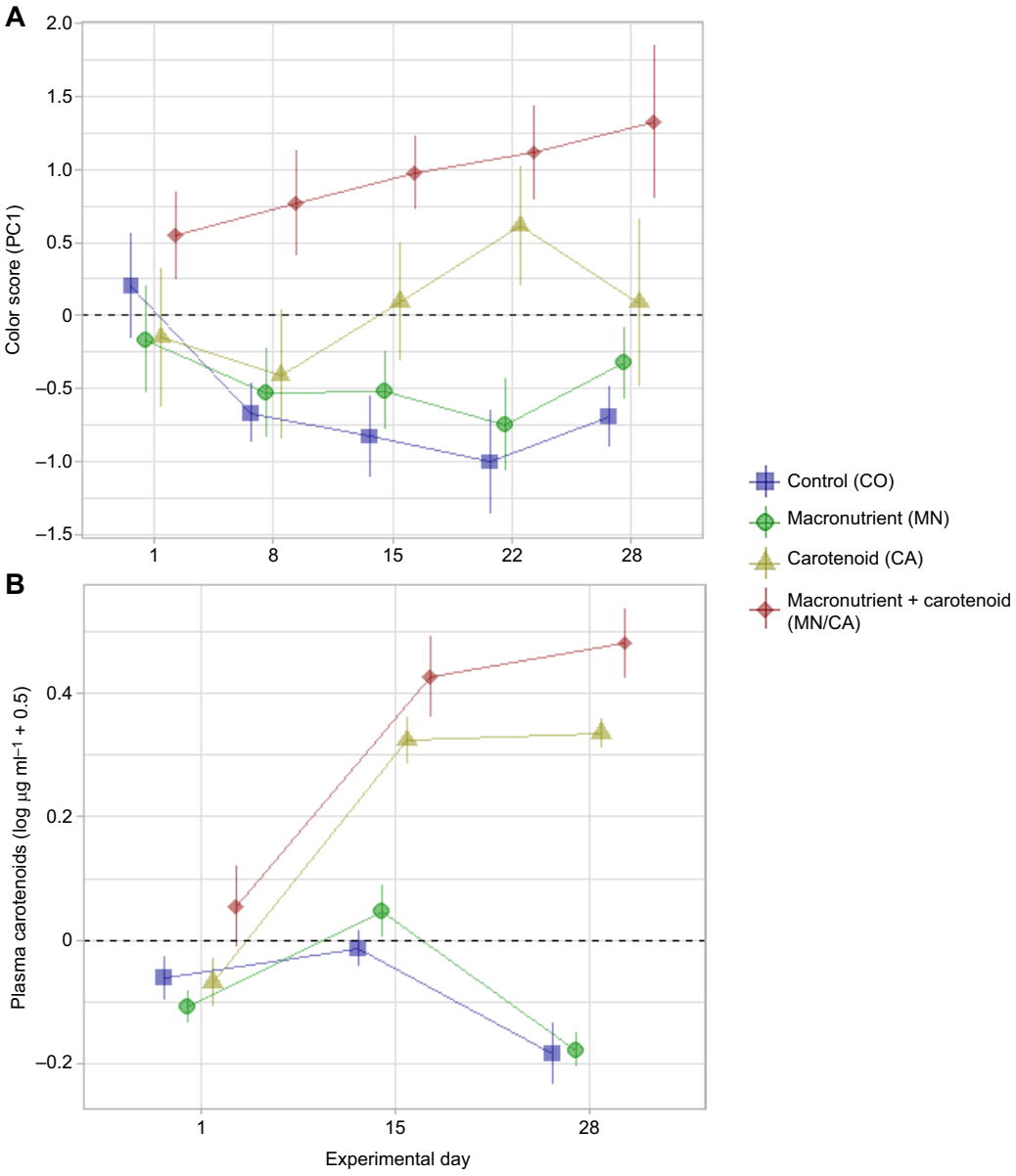

Fig. 2. Change in eye patch coloration and plasma carotenoid levels over the experiment period (day 1 to 28) in each treatment group. (A) Eye patch coloration (PC1 scores). (B) Plasma carotenoid levels (log µg ml^{-1}+0.5). Data are presented as means±s.e.m. (n=10 in each treatment group).

experiment, carotenoid concentrations differed across groups significantly (LMM, day 28: $F_{3,36}$=69.33, P<0.0001), with concentrations increasing in carotenoid-supplemented groups (LMM, days 1–28: MN/CA, $F_{1,9}$=25.83, P<0.0001; CA, $F_{1,9}$=130.24, P<0.0001) and decreasing in non-supplemented groups (LMM, days 1–28: CO, $F_{1,9}$=7.44, P=0.02; MN, $F_{1,9}$=4.43, P=0.065). At day 28, carotenoid levels in MN/CA and CA groups were significantly higher than in CO and MN groups (P'<0.0001 in all comparisons). Levels in MN/CA birds tended to be higher than in CA birds (P'=0.079). The amount of carotenoid ingested also had no effect on carotenoid levels in these two treatment groups (Table 1).

Plasma carotenoids also differed as a function of sex and time (Table 1, Fig. 3). No differences in plasma carotenoid levels were found between males and females over the experiment period (LMM, day 1: $F_{1,38}$=2.10, P=0.16; day 28: $F_{1,38}$=0.01, P=0.91). However, plasma carotenoid concentrations significantly increased over time in males (LMM, days 1–28: $F_{1,22}$=7.49, P=0.01), while concentrations in females did not significantly change (LMM, days 1–28: $F_{1,16}$=3.12, P=0.09).

Change in nutritional intake

Body mass had no effect on nutrient consumption (Table 1). MN/CA mynas (36.3% P, 27.1% L, 36.6% C) ingested significantly more L (P'<0.0001) and less C (P'<0.0001) than CA and CO birds (32.4% P, 15.6% L, 52% C). Relative consumption of macronutrients also differed between the sexes significantly (Table 1, Fig. 4; for all groups, see Fig. S4). In MN/CA females (35.2% P, 21.1% L, 43.7% C), intake levels of P (P'=0.96), L (P'=0.6) and C (P'=0.17) were not different than in CA and CO females. In contrast, MN/CA males (37.5% P, 33% L, 29.5% C) consumed significantly more L (P'<0.0001) and less C (P'<0.0001) than CA and CO males. Within the MN/CA group, females consumed more C (P'=0.02) and tended to consume less L (P'=0.065) than males.

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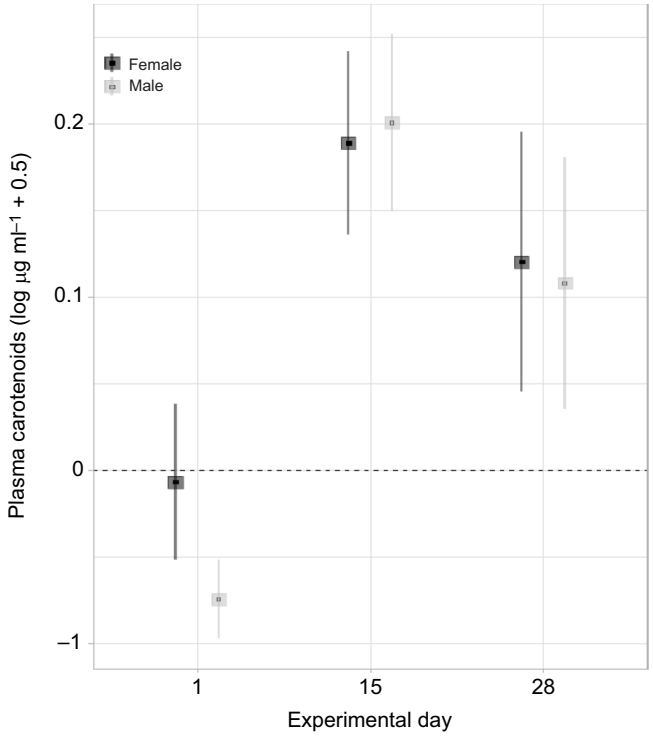

Fig. 3. Change in plasma carotenoid levels (log μg ml^{-1}+0.5) over the experiment period in each sex across all treatment groups. Data are presented as means±s.e.m. (*n*=17 females; *n*=23 males).

Effects of specific macronutrient intake

GAMs showed the effect of C intake and an interaction between P and L intake on color scores (Table 2). Low C intake as well as high L–low P intake were associated with higher color scores (Fig. 5A). Moreover, L intake had a positive effect on plasma carotenoid concentrations, with high L intake associated with higher levels of plasma carotenoids (Table 2, Fig. 5B).

DISCUSSION

Overall, our results support the prediction that carotenoids and macronutrients jointly influence eye patch coloration, while also suggesting that male and female mynas make different dietary choices with respect to maximizing signal coloration.

We demonstrated that carotenoid supplementation alone is not sufficient to produce a carotenoid-based signal. Indeed, only mynas supplemented with both macronutrients and carotenoids (MN/CA) displayed a significantly more colorful eye patch compared with non-supplemented birds (CO and MN), whereas no differences were found between carotenoid-supplemented (CA) and non-supplemented birds. In carotenoid-supplemented mynas, plasma carotenoid levels significantly increased compared with unsupplemented birds. However, we also found that MN/CA birds had marginally higher levels of plasma carotenoids than CA birds. It seems that mynas with the opportunity to self-select their macronutrient intake tended to have higher plasma carotenoid levels, suggesting that macronutrients might play an important role improving carotenoid absorption. Demonstrating a joint effect of carotenoids and macronutrients sheds light on why food deprivation can also reduce coloration.

The differences between treatment groups in coloration and plasma carotenoid concentration indicate that, although the ingestion of dietary carotenoids is essential to increase plasma carotenoid levels and produce coloration, specific macronutrient intake plays a central role in carotenoid metabolism and signaling in mynas. Indeed, as predicted, MN/CA birds ingested more L and less C than birds fed dog pellets alone. Further analyses revealed that high L, low C and low P intakes were indeed associated with higher color scores and plasma carotenoid concentrations, thus highlighting the fundamental role of macronutrient selection on modulating carotenoid-based coloration in birds.

As a major source of energy, C could likely fulfill potential energetic requirements associated with the production of colored signals (Hill, 2000, 2002; McGraw et al., 2005; Shawkey et al., 2006). Recent advances on the mechanistic links between carotenoid signals and individual quality have highlighted the role played by mitochondrial function (Cantarero et al., 2020a,b; Hill et al., 2019). Coloration based on red carotenoids (ketocarotenoids) requires the conversion of yellow carotenoids via ketolation in the inner mitochondrial membrane, which makes coloration reliant on mitochondrial function efficiency (Cantarero et al., 2020a,b; Hill et al., 2019). This could explain why the link between color display and individual condition has been found to be stronger in bird species that rely on carotenoid bioconversions to produce their signals (Weaver et al., 2018). In this scenario, producing carotenoid-based signals could generate a demand for high-energy food sources in order to sustain mitochondrial energy production. However, as

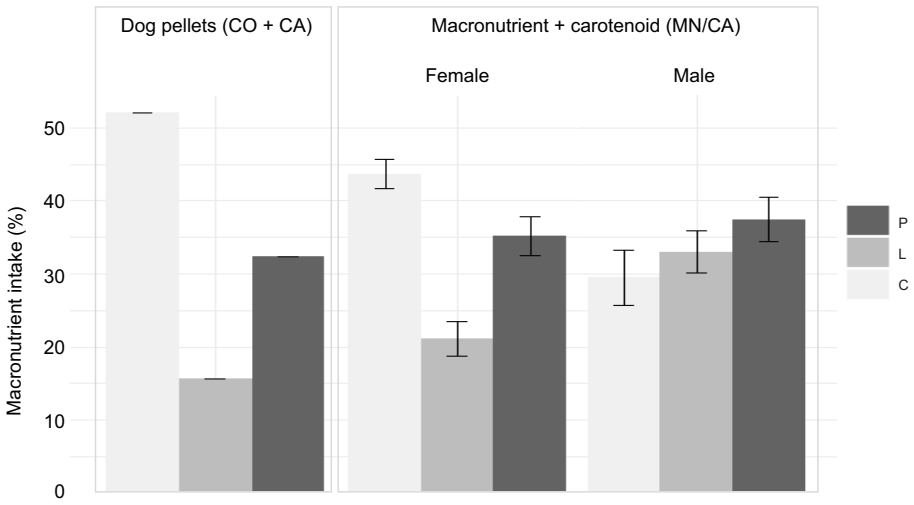

Fig. 4. Percentage of weekly protein (P), lipid (L) and carbohydrate (C) intake in control (CO), carotenoid (CA) and macronutrient+carotenoid (MN/CA) groups in female and male common mynas. CO and CA birds were provided with the usual dog pellet diet and could not adjust their relative intake of each of the macronutrients. MN/CA birds were offered a free choice between P, L and C artificial pellets and were therefore free to adjust their relative intake of the three macronutrients. Data are presented as means+s.e.m. (*n*=10 in each treatment group).

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Table 2. Results of generalized additive models examining the relationships between intake of a specific nutrient or combination of nutrients with coloration and plasma carotenoid levels in common mynas

Response	Predictor	d.f.	*F*	*P*
Color scores (PC1) – all groups	Protein	24	0.00	0.14
	Lipid	24	0.00	0.55
	Carbohydrate	24	**0.22**	**0.02**
	Protein×Lipid	22	**0.70**	**0.004**
	Protein×Carbohydrate	22	0.00	0.21
	Lipid×Carbohydrate	21	0.00	0.38
Plasma carotenoid levels – all groups	Protein	13	0.00	0.52
	Lipid	13	**0.34**	**0.01**
	Carbohydrate	13	0.00	0.22
	Protein×Lipid	11	0.00	0.66
	Protein×Carbohydrate	11	0.69	0.10
	Lipid×Carbohydrate	10	0.00	0.63

P-values for significant factors (α<0.05) are shown in bold.

per our prediction, we found a negative association between carbohydrate intake and signal production in mynas. Although this does not necessarily mean that color production in mynas does not generate energetic costs, it is possible that the yellow coloration in this species is not produced by metabolic conversion of carotenoids but simply by the deposition of dietary pigments into the skin (Weaver et al., 2018), yet this remains to be further explored.

Contrary to C, P and L both play a direct role in producing colored signals. P forms light-reflecting nanostructures made up of collagen or keratin onto which carotenoid pigments are deposited (Prum and Torres, 2003; Shawkey and Hill, 2005). The presence of nanostructured tissues typically results in the production of colors such as UV, blue, green, white or iridescent (Hill and McGraw, 2006). As our results are consistent with our prediction that relative P intake should be lower in birds displaying enhanced carotenoid-based coloration, we suggest that P intake is more likely to be of importance to colored displays produced essentially by nanostructures, such as the UV/blue tail feathers of blue tits (Peters et al., 2007). As expected, we also found that more colored birds in the MN/CA treatment consumed a higher proportion of L in their diet. Previous work has shown that the presence of dietary L and the production of lipoproteins are directly linked to carotenoid absorption and transport (McGraw and Parker, 2006; Toomey et al., 2017). When carotenoids are absorbed, they are first incorporated into micelles, which are formed from L, before moving through the intestines and bonding to lipoproteins that will transport them through the plasma (During and Harrison, 2004; Erdman et al., 1993; Parker, 1996). The dietary intake of L is crucial to this process as it stimulates bile flow, which allow the formation of micelles. Therefore, low levels of L could reduce carotenoid absorption and transport and directly affect signal expression (Erdman et al., 1993; McGraw and Parker, 2006). It is also possible that individual's digestive efficiency and lipid absorption capabilities could have an impact on signal quality, as

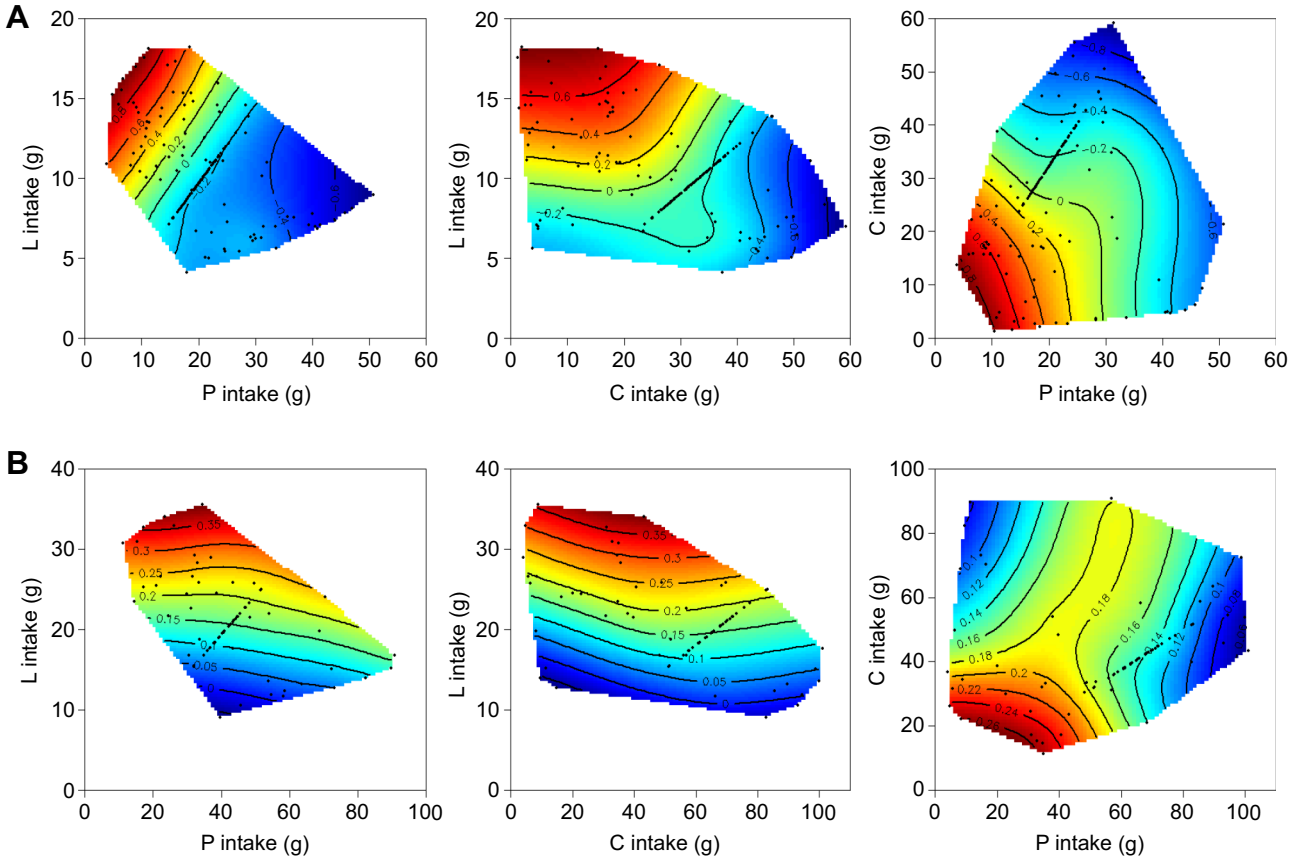

Fig. 5. Effect of macronutrient intake on color score and plasma carotenoid concentration in common mynas. Response surfaces show how relative macronutrient intake in grams [protein (P), lipid (L) and carbohydrate (C)] affects (A) color scores (PC1) and (B) plasma carotenoid concentration (log μg ml^{-1}+0.5). Points on the surface indicate an individual bird (n=40). For all surfaces, red regions indicate the highest values, i.e. highest color scores (A) or highest plasma carotenoid concentrations (B), which decrease as the colors shift to yellow, then blue. Contour lines within each surface are isolines indicating equal values for each variable along that line.

higher levels of lipid absorption would allow for more efficient absorption and transport of carotenoids (Madonia et al., 2017). Although it is important to note that plasma carotenoid levels do not always predict coloration (Koch et al., 2016), ingestion, absorption and transport of carotenoids are still indispensable to color production. This could explain the significant differences observed in eye patch coloration between the MN/CA and CO groups and the similarities in coloration between CA and CO groups. Indeed, MN/CA mynas ingested a higher proportion of L than CA birds, which could have improved the absorption and transport of carotenoid pigments, ultimately facilitating signal production.

Our results also showed that macronutrient intake, plasma carotenoid levels and, to some extent, coloration, differed across sexes. Males showed a significant increase in plasma carotenoid levels over the experiment, with the most colorful individuals observed in the MN/CA group in comparison with birds from CO and MN groups. The lack of interaction between sex and treatment suggests that carotenoid supplementation may not be the only reason behind the significant rise of carotenoid levels in males throughout the experiment. If an interaction was present, higher carotenoid absorption in males would have more likely explained the differences in plasma carotenoid levels between males and females. However, it is possible that this increase in males could be linked to the release of carotenoid pigments from internal storage. Mynas were tested during the beginning of their breeding season, when we expected signal production to be maximized. When the need for signal production is high, and males are also unable to obtain carotenoids from their diet, it may be possible that carotenoids are released into the blood from the liver or the adipose tissue (García-de Blas et al., 2015; Goodwin, 1950).

Supplemented males also consumed proportionally more L and less C than CA birds and MN/CA females. The macronutrient intake of males was high L, low P and low C and may be a likely factor improving carotenoid absorption during digestion, facilitating transport through the bloodstream, and increasing signal coloration. These results suggest that males could ingest more L in the presence of dietary carotenoids to facilitate carotenoid uptake for transport to and/or deposition in the integument. Mynas are known for selecting foods and macronutrients based on their nutritional needs, even when no perceptual signatures such as olfactory and/or gustatory cues are present (Machovsky-Capuska et al., 2016a; Peneaux et al., 2017). It is also known that males can select food items with high carotenoid content during feather production (Senar et al., 2010; Walker et al., 2014). However, we now show that male mynas can select specific combinations of macronutrients for their color-enhancing properties.

As opposed to males, no significant effects were observed on plasma carotenoid concentrations and coloration in females during the experiment. A similar macronutrient intake was also observed in females from the MN/CA, CA and CO groups. The absence of change in female coloration is consistent with sexual selection theory, which states that signals are often relatively more pronounced in males in response to females assessing the fitness of potential mates based on their coloration (Andersson and Simmons, 2006; Darwin, 1871; Winterbottom, 1929). Our results are aligned with these suggestions, showing that females in the MN/CA treatment consumed a balance of macronutrients (low L, high P and high C) that is associated with low levels of plasma carotenoids and poor coloration. Because females ate high proportions of C and P, they had to consume relatively less L, which could be linked to low carotenoid absorption and transport as well as poor signal coloration.

Sex-specific macronutrient foraging strategies have been previously observed in free-ranging birds (Machovsky-Capuska et al., 2016b). In our study, females in the MN group consumed more P than males (Fig. S4) in a pattern likely related to differences in post-ingestive nutrient processing and reproductive performance (Maklakov et al., 2008). A visual assessment post-experiment revealed an enlargement of the gonads, which confirmed that mynas were preparing for reproduction. In reproductive females, growth of the oviduct and accretion of egg P, in addition to normal maintenance, generate a strong drive for P-rich food (as a supply of essential amino acids, glucose, L, energy and vitamins) (Murphy, 1994). With the higher requirements for P during egg production, a change in nutritional preference called a ‘protein shift’ is often observed (Brice, 1992; Krapu and Reinecke, 1992; Yamane et al., 1979). Thus, females likely maximize their nutritional intake in favor of reproductive functions rather than signal production.

In sum, we found that both the intake of macronutrients and carotenoid pigments are essential to the production of carotenoid-based displays. The consumption of specific macronutrient combinations can impact carotenoid metabolism directly by facilitating or reducing pigment absorption and transport, thus modulating the amount of carotenoid available for coloration. We also showed that individuals were able to detect and select specific macronutrients in relation to their nutritional needs. Indeed, we identified potential sex-specific nutritional strategies, with females maximizing nutrient intake for reproductive functions and males preferring macronutrient combinations that increased pigment absorption for signal expression. Here, we highlighted the importance of adopting a nutritionally integrated approach to carotenoid-based signaling, which encompasses carotenoid availability as well as intake of other essential macronutrients. These findings also raise awareness on the effects of impoverished diets and malnourishment on signal expression. More specifically, in urban habitats, the abundance of anthropogenic foods and lack of natural resources promote nutritionally unbalanced environments and lead to impoverished diets (Coogan et al., 2018; Peneaux et al., 2021). Such changes in nutritional conditions have the potential to affect color signaling and sexual selection, ultimately influencing reproductive success and population demography.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.P., G.M.C., A.G.; Methodology: C.P., G.M.C., J.E., A.G.; Software: J.E.; Validation: A.G.; Formal analysis: C.P., G.M.C., A.G.; Investigation: C.P., A.G.; Resources: G.M.C., J.E., A.G.; Data curation: C.P., G.M.C., A.G.; Writing - original draft: C.P., G.M.C., A.G.; Writing - review & editing: C.P., G.M.C., J.E., A.G.; Visualization: C.P., A.G.; Supervision: G.M.C., A.G.; Project administration: A.G.; Funding acquisition: A.G.

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Data availability
Raw data from this study are available in Mendeley Data: doi: 10.17632/djpymvcp2m.1

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SUPPLEMENTARY MATERIAL

Table S1. Nutritional content of the standard captivity diet (dog pellets) for mynas.

Ingredient (%)	**SUPERCOAT® Puppy With Real Meat**
Crude Protein	29.00
Crude Fat	14.00
Salt (NaCl)	1.80 (max)
Crude Fiber	6.00 (max)
Omega Fatty Acids	1.40 (min)
DHA	0.05 (min)

Table S2. Ingredients and calculated nutrient composition of the three foods provided during the experiment (from Machovsky-Capuska et al., 2016).

Ingredient (%)	**High Protein**	**High Lipid**	**High Carbohydrate**
Corn starch	-	-	42
Corn gluten meal	40.00	-	-
Meat and bone meal	10.00	-	-
Poultry by-product meal	20.00	-	-
Powdered cellulose	23.96	63.52	39.26
Soy protein concentrate	1.28	-	-
Poultry fat	-	15.61	-
Soy oil	2.62	15.00	-
Sodium chloride	-	0.37	0.30
Sodium bicarbonate	0.16	0.28	0.27
Potassium bicarbonate	0.60	1.04	1.28
Potassium sulphate	0.50	0.75	0.50
DL Methionine	-	-	-
Lysine HCL	-	-	-
Threonine	-	-	-
Limestone	0.38	0.95	0.91
Monodicalcium phosphate	-	1.98	2.00
Vitamin/Mineral Premix*	0.50	0.50	0.50
Metabolizable energy (kcal/kg)	2600.00	2600.00	2600.00
Crude Protein	42.00	0.21	0.42
Total Calcium	0.90	0.90	0.90

Total Phosphorus	0.53	0.42	0.43
Digestible Phosphorus	0.40	0.40	0.40
Digestible Lys	1.27	0.00	0.00
Digestible Met+Cys	1.36	0.00	0.00
Digestible Thr	1.34	0.00	0.00
Sodium	0.22	0.22	0.22
Potassium	0.58	0.58	0.58
Chloride	0.23	0.23	0.23

*dosed to supply per kg: ethoxyquin, 100 mg; biotin, 0.2 mg; calcium pantothenate, 12.8 mg; cholecalciferol, 60 µg; cyanocobalamin, 0.017 mg; folic acid, 5.2 mg; menadione, 4 mg; niacin, 35 mg; pyridoxine, 10 mg; trans-retinol, 3.33 mg; riboflavin, 12 mg; thiamine, 3.0 mg; dl-α-tocopheryl acetate, 60 mg; choline chloride, 638 mg; Co, 0.3 mg; Cu, 3 mg; Fe, 25 mg; I, 1 mg; Mn, 125 mg; Mo, 0.5 mg; Se, 200 µg; Zn, 60 mg.

Figure S1. Standardized photographs of a bird's eye patch at (A) capture and (B) after eight weeks in captivity.

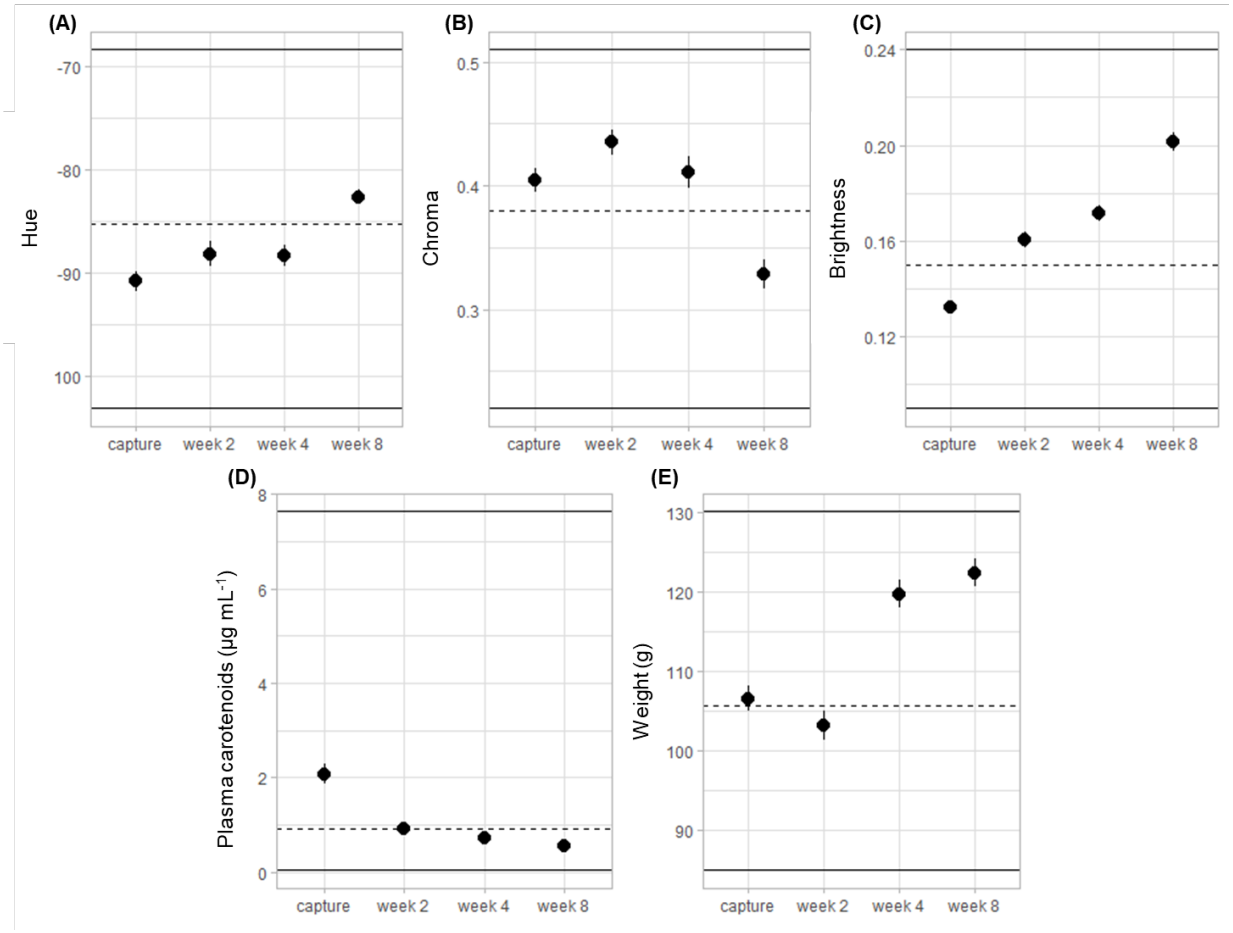

Figure S2. Change in coloration, plasma carotenoid levels and weight during the fading period (mean ± standard error). Measurements were taken at capture and following 2 weeks, 4 weeks and 8 weeks in captivity. Hue (A), chroma (B) and brightness (C) are standard parameters scored to quantify coloration. Hue and brightness increased over time, while chroma decreased – taken all together, this show a decrease in eye patch coloration over time. Plasma carotenoid levels (D) also decreased during time in captivity. Individual body weight (e) increased after a month in captivity. The dotted line represents average population value under natural conditions while the solid black lines show the maximum and minimum values found in populations of common mynas (C Peneaux, unpublished data).

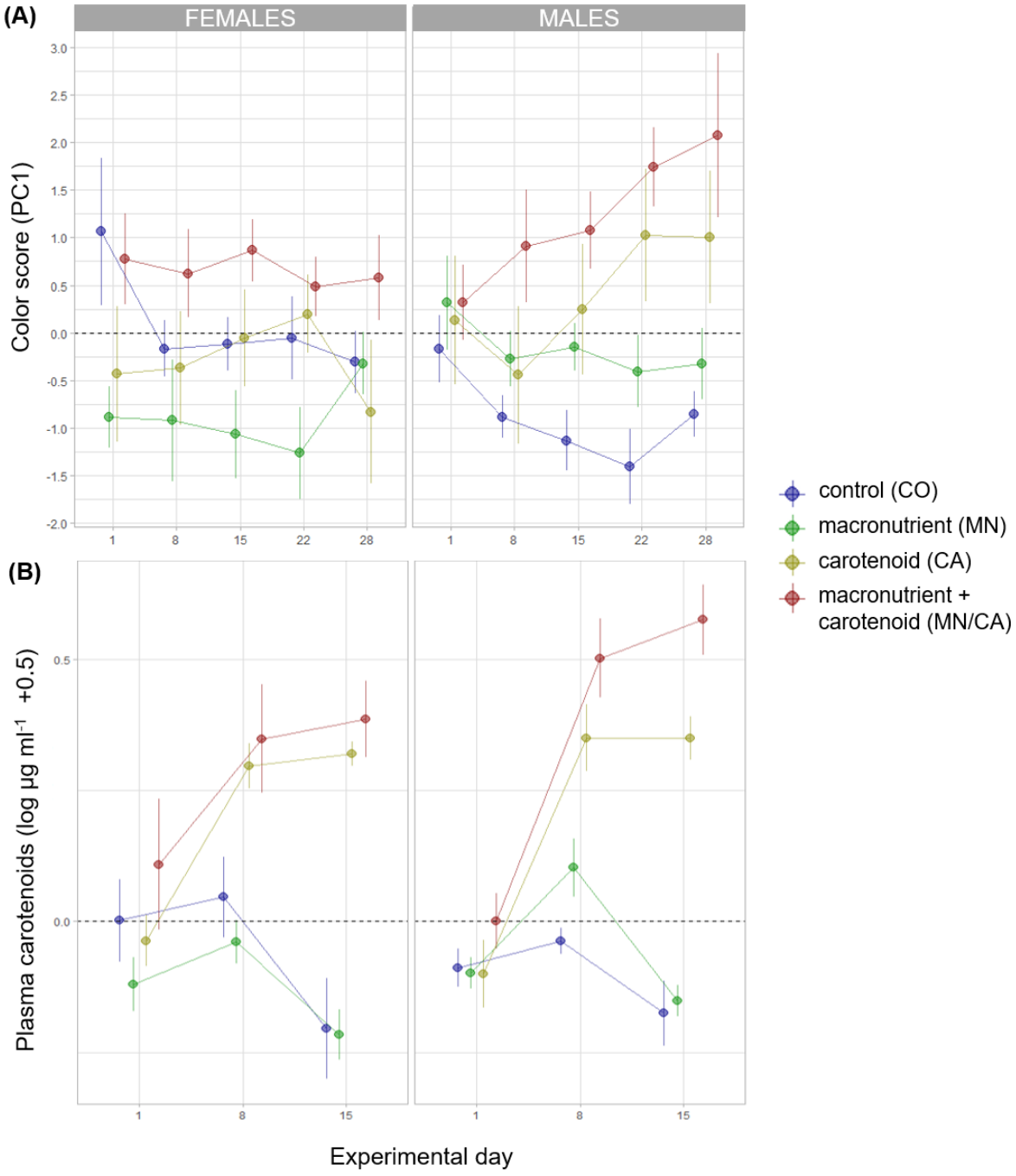

Figure S3. Change in eye patch coloration (A) and plasma carotenoid concentrations (log µg ml^{-1} +0.5) (B) over the experiment period in females (F) and males (M) for each treatment group (mean ± standard error).

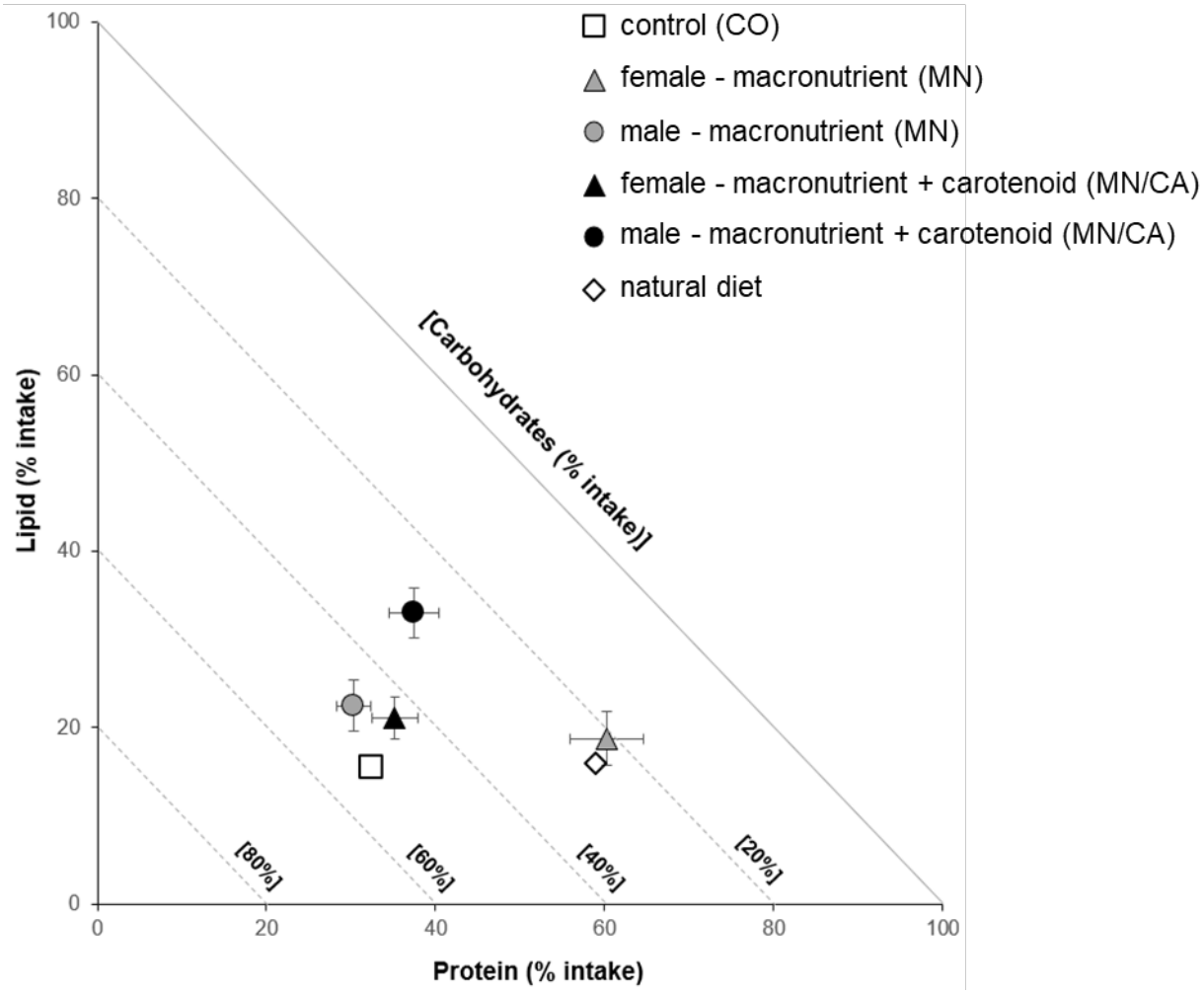

Figure S4. Proportion-based nutritional geometry plots showing the mean (± standard error) diet balance of macronutrient intake (protein, lipid and carbohydrates) selected by male and female mynas from free choice food groups (macronutrient and macronutrient + carotenoid) and dog pellets (control and carotenoid). Natural diet of mynas in the wild (see Machovsky-Capuska et al., 2016) was also included. Nutritional intake in MN males and MN/CA females did not differ from control birds. MN females ingested nutrients in proportions similar to the ones of wild birds, characterized by high protein intake. MN/CA males consumed a high proportion of lipids compared to other groups. To geometrically define diets, % Protein is plotted against % Lipid. Considering that the three macronutrients in the mixture ingested sum to 100%, plotting % Protein (first axis) and % Lipid (second axis) will automatically reflect the value of % Carbohydrate in the third axis (Raubenheimer, 2011).