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Do glucocorticoids or carotenoids mediate plumage coloration in parrots? An experiment in *Platycercus elegans*

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ABSTRACT

Conspicuous coloration can indicate phenotypic quality, and may reflect exposure or vulnerability to stress, or access to essential nutrients such as pigments. Although the production of pigmented colours is well understood, much less is known about how structural colours are affected by physiological state. In this study, we tested whether glucocorticoids (corticosterone) predicted expression of plumage coloration in an Australian parrot, the crimson rosella (*Platycercus elegans*). Parrots provide an interesting and unique test, as they possess conspicuous coloration produced by distinctive pigments known as psittacofulvins, in addition to structural coloration. We have previously documented that coloration in *P. elegans* is condition-dependent and responds to dietary manipulation. Here, n = 21 *P. elegans* underwent a dietary manipulation (including food restriction or carotenoid supplementation) during which they moulted, and the change in reflectance was measured for three structural and three pigmentary plumage patches. Stress-induced corticosterone (10 min after handling) measured at the start of the experiment predicted change in coloration in two pigmentary patches (crown and front). We also found that change in stress-induced corticosterone during the experiment was associated with the change in coloration of the crown and two structural patches (cheek and epaulette). Baseline corticosterone (< 3 min after handling) was not associated with any measure of coloration. We found no effects of dietary manipulation on baseline or stress-induced corticosterone, but carotenoid supplementation was associated with an increase in a measure of chronic stress (heterophil/lymphocyte ratio), and the corticosterone response to handling decreased over the course of the study. Our results suggest that corticosterone may be linked to colour expression more broadly than previously recognised, including psittacofulvin and structural coloration in parrots, and they confirm the independence of plumage pigmentation in parrots from carotenoid accumulation. Moreover, our study provides new insight into the stress responses of Psittaciformes, one of the most highly threatened avian orders.

1. Introduction

Conspicuous, condition-dependent signals such as elaborate coloration can reflect various aspects of phenotypic quality (Hill, 2006; Hill and Hill, 2002). For example, colour traits may convey information about the condition or physiological state of the bearer, access to essential nutrients such as pigments and antioxidants (e.g. carotenoids), or reflect exposure or vulnerability to stress (Lendvai et al., 2013; McGraw and Ardia, 2003; McGraw et al., 2002). Many mechanisms are involved in the production of animal coloration, but the two main types are pigments (such as carotenoids and melanins) and variation in integument structure (Mcgraw, 2006). Both carotenoid pigments and structural coloration (though the latter has been studied less) have been widely implicated in condition-dependence signalling (e.g. Dale et al., 2001; Hamilton and Zuk, 1982; Hill, 2006), but the role of physiological response to stress is less clear.

Biologically meaningful stressors may come in many forms, such as nutritional deficits, immune challenges, oxidative, and behavioural challenges, and most of these have been linked to signal expression in animals (e.g. Mougeot et al., 2010; Romero and Wingfield, 2015; Roulin et al., 2011; Roulin et al., 2008). One of the primary means of assessing stress is by quantifying circulating levels of the glucocorticoids which

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mediate the endocrine response to environmental stress in vertebrates (e.g. Sapolsky et al., 2000). Corticosterone (CORT) is the primary glucocorticoid in birds and may negatively affect an individual's condition during chronic stress and can induce immunosuppression (Buchanan, 2000; Cote et al., 2010a,b; Moore and Miller, 1984; Sapolsky et al., 2000; Tokarz, 1987).

CORT may be associated with the production or deposition of the pigments such as melanins or carotenoids (e.g. Almasi et al., 2010; Loiseau et al., 2008; Martínez-Padilla et al., 2013; Roulin et al., 2008), which form the basis of many well-studied colour signals (Hill, 2006; Hill, 2011). CORT also inhibits protein deposition (Romero et al., 2005; Sapolsky et al., 2000), and in birds higher CORT has been linked to reduced feather structural quality (DesRochers et al., 2009; Grunst et al., 2015; Jenni-Eiermann et al., 2014; Lattin et al., 2011). As such, there is a plausible link between CORT and feather barb nanostructure, and thus structural coloration in birds (Kennedy et al., 2013). CORT is also known to be directly affected by dietary restriction (e.g. Lendvai et al., 2014). Therefore, CORT is a plausible candidate in mediating links between diet, stress and at least pigment- or structural-based coloration, and thus the information content of many condition-dependent colour signals. However, the relationships between CORT and colour traits seem to vary, even within the same colour mechanism. Recent research provides support for negative, positive, or no relationships between CORT levels and carotenoid or melanin coloration (e.g. Fairhurst et al., 2014; Grunst et al., 2015; Jenkins et al., 2013; Kennedy et al., 2013; San-Jose and Fitze, 2013). Compared to pigmentary coloration, the relationships between CORT and structural coloration have been much less studied, but current evidence for any such associations is similarly mixed (Grindstaff et al., 2012; Henderson et al., 2013).

In this study, we extend tests of the associations between CORT and plumage coloration to both structural coloration and utilize the unique, little studied pigmentary colour system, based on psittacofulvins, afforded by parrots (order Psittaciformes). Parrots are a large, phylogenetically distinct lineage of birds, which feature some of the most striking coloration found in nature and an extremely high proportion of threatened species (Berg and Bennett, 2010; Delhey, 2015; Hastad and Odeen, 2008; IUCN, 2016; Juniper and Parr, 1998; Mcgraw, 2006; Nemesio, 2001). Although the unusual mechanism of parrot coloration has been recognised for over a century (Krukenberg, 1882), only relatively recently have the pigments involved in their colour production been elucidated (Cooke et al., 2017; Mcgraw, 2006; McGraw and Nogare, 2005; Stradi et al., 2001). This work has revealed that much parrot coloration is based on a unique class of polyenal lipochrome pigments called psittacofulvins. In contrast to the carotenoid pigments of most other bird species, it is thought that psittacofulvins are synthesized endogenously during feather growth, most likely within the follicular tissue at the site of growing feathers (McGraw and Nogare, 2004). Parrots also produce coloration rich in short wavelengths (UV and blue) produced by feather nanostructure (Berg and Bennett, 2010; Dyck, 1971; Mullen and Pohland, 2008; Prum, 2006; Prum et al., 1999).

Despite their remarkable coloration, popularity and perilous conservation status, little is known about the stress responses of parrots, their utilisation of carotenoids, or the functional significance of their coloration (Berg and Bennett, 2010; Cooke et al., 2017; Knott et al., 2010; McGraw, 2006; McGraw and Nogare, 2004; Ribot et al., 2019). However, there are several reasons to hypothesize that either form of parrot coloration (pigment or structural) could function as condition-dependent signals. Firstly, psittacofulvins act as powerful antioxidants (McGraw, 2005; Morelli et al., 2003), and the metabolic processes and enzymatic pathways involved in psittacofulvin synthesis may be sensitive to limitation in intake of energy or particular nutrients. This suggests that psittacofulvins may provide signals of quality in a similar way to other forms of pigmentary coloration (Masello et al., 2008; Masello et al., 2004; Masello and Quillfeldt, 2003). Further, evidence suggests that the mechanism of non-iridescent structural coloration used by parrots can provide sexually selected signals of quality in passerine birds (e.g. Amundsen et al., 1997; Andersson et al., 1998; Keyser and Hill, 1999; Peters et al., 2007; Siefferman and Hill, 2005). In burrowing parrots (*Cyanoliseus patagonus*), the size of a psittacofulvin-based plumage patch covaried with a measure of body condition in adult males; the size and luminance (achromatic reflectance) of the same patch, as well as luminance of a structural patch, varied between years with different levels of precipitation (indirectly suggesting a possible link with food availability); and luminance of a structural patch in nestlings was related to body weight (Masello et al., 2008; Masello and Quillfeldt, 2003).

As parrots use psittacofulvin pigments rather than carotenoids directly in their feathers, the prevailing, yet hitherto untested, assumption is that dietary carotenoid intake will have no effect on their coloration, as it does many other taxa. However, parrots do accumulate high levels of circulating carotenoids (McGraw and Nogare, 2004), which are sensitive to dietary intake (Knott et al., 2010). As such, this hypothesis warrants testing to eliminate the possibility that carotenoids indirectly affect psittacofulvin-based coloration via general condition effects, physiological trade-offs between the deposition of psittacofulvins in feathers and the intake and utilisation of carotenoids, or through a role as pigment precursors. To date the relationships between CORT, carotenoids, diet, and immune function or condition remain untested in parrots, and whether stress or dietary intake is associated with the expression of parrot plumage coloration is unknown.

Here, we studied crimson rosellas (*Platycercus elegans*), a parrot native to south-eastern Australia which possesses multiple patches of both pigment- and structurally-based plumage coloration (Berg and Bennett, 2010; Ribot et al., 2019). Birds were assigned to one of three diet treatments (food restriction to lower energy intake; carotenoid supplementation; and controls), and we considered multiple plumage patches comprising two forms of colour production (psittacifulvin- or structural-based plumage). We have previously shown that food restriction resulted in a decrease in body weight and carotenoid supplementation resulted in higher levels of circulating carotenoids (Knott et al., 2010). Moreover, we have found that plumage coloration following moulting during this experiment was affected by the dietary treatment, particularly for structural coloration (unpublished results). Our aim in the study reported here, was to test whether physiological indices of stress were associated with the diet manipulation, and whether circulating CORT or carotenoids measured prior to or after the experiment predicted the change in plumage coloration of birds that underwent the manipulation. Specifically, we tested: (i) how baseline (collected within 3 min of disturbance) and stress-induced (collected after 10 min handling/restraint) CORT responded to dietary manipulation; (ii) whether CORT, or the change in CORT over the course of the experiment, predicted the change in plumage reflectance following moult during the experiment; (iii) whether circulating carotenoids were related to parrot coloration; and (iv) whether additional measures of chronic stress (heterophyl/lymphocyte ratio) or immunocompetence (phytohemagglutinin-induced response) were affected by the dietary manipulation or covaried with CORT in parrots.

2. Methods

2.1. Experimental design

We carried out the experiment using 21 captive *P. e. elegans*, from early spring (20 March 2007) to late autumn (12 November 2007) to ensure that the experiment encompassed the putative moulting period for *P. elegans*. In Australia, wild *P. elegans* populations exhibit signs of moult over a period of up to six months, from late spring (November) to mid autumn (April); outside these months Higgins (1999) found no birds with signs of tail or primary moult and only 4.1% of birds with signs of active body moult (n = 121). Our observations of active moult (presence of developing feathers) on the head, breast/belly, back and

tail of captive *P. e. elegans* housed outdoors in the U. K. in 2006–2007 confirmed that moult took place mainly during summer (unpublished results).

Captive-bred birds were acquired from breeders in the U.K., transported to the University of Bristol and housed individually in 21 outdoor aviaries (ca. 1.5 × 1 × 2 m high, part-shaded by garden netting and with access to natural light) prior to and during the experiment. The birds underwent a dietary manipulation treatment, as part of another study on vision, as described in Knott et al. (2010). Briefly, individual aviaries, each holding one bird, were allocated to 'blocks' of three, with one aviary per block randomly assigned to each of the three diet treatments. Each block contained the same sex (three male blocks, four female blocks), and individuals were randomly assigned to aviaries. There was an initial pre-experimental 'wash-in' period of four weeks, during which all birds were fed an *ad libitum* seed mix comprising one part Barrow Mill 'Parrot' mix (with peppers and dried fruit removed) and two parts Barrow Mill 'Parakeet/cockatiel' mix (Barrow Mill, Bristol), plus two unshelled peanuts and 10 g Granny Smith apple per day; this diet was designed to mimic the natural diet of predominately seeds, nuts and fruit, and is a standard captive maintenance diet for this species (Forshaw and Cooper, 2002). After this wash-in, the experimental period began (20 March to 4 April, with each block commencing on a different day), and birds were then started and maintained on one of the following experimental treatments: (i) 'control' was the maintenance diet described in part (a) with *ad libitum* unmanipulated water; (ii) 'carotenoid' was the described maintenance diet plus an *ad libitum* lutein/zeaxanthin carotenoid supplementation added to tap water at 100 μg/ml (Oro Glo-8, Kemin Agrifoods Europe, Herentals, Belgium); (iii) food 'restricted' was 38 g of a mix comprising one part maintenance diet plus four parts oat husk (J. E. Haith Ltd, Grimsby) by volume (4:3 seed:husk by weight) with unmanipulated water. Food and water were replaced daily until the birds were sacrificed. Lutein and zeaxanthin are dietary carotenoids used to colour feathers in birds and found in high concentrations in the blood of parrots (McGraw and Nogare, 2004). As expected, by week 12 birds receiving the carotenoid treatment had significantly elevated circulating carotenoid concentrations (see below), and birds receiving the food restriction treatment had lower body weight evident by week 4 (Fig. S1; Knott et al., 2010).

2.2. Sampling and data collection

All birds were weighed twice a week using a spring balance (Pesola AG, Switzerland), to monitor weight changes during the experiment, as reported in Knott et al. (2010) and summarised (weights every 4 weeks for the first 12 weeks during diet manipulation) in Fig. S1. At week 0, 4 and 12 after the diet manipulation commenced, birds were captured, blood sampled within three minutes after capture, and again after being held in a cotton bag for 10 min. The three birds in each experimental block were sampled at the same time. Blood was collected in 75ul capillary tubes before being transferred to 1.5 ml microcentrifuge tubes. Blood samples were used to assay circulating CORT and carotenoid levels, and week 12 blood samples were also used to estimate heterophil/lymphocyte ratios (as described below) and for DNA sexing (following Eastwood et al. (2015), Griffiths et al. (1998)). For a subsample of three female rosellas (one from each treatment group in the same block), we also collected four consecutive blood samples (1 min, 10 min, 30 min and 50 min after capture with birds held in identical cotton bags in a darkened environment between samplings) to assess within individual changes in CORT over time to a standardised stressor (handling); this sampling was carried out 10 August 2007 (58 days after the third blood sampling at week 12; during which time the birds were still held on the same experimental diets).

2.3. Stress and immune measures

For each bird we measured circulating CORT (ng/ml) at 'baseline' (i.e. blood sampled < 3 min after capture) and 'stress-induced' (i.e. blood sampled 10 min after capture/handling) levels, following the standard capture-restraint protocol used in studies analyzing the CORT response (Wingfield, 1994). The blood was centrifuged at 11,000g for 15 min and the plasma frozen. CORT concentrations were measured after extraction of 20 μl aliquots of plasma in diethyl ether, by radioimmunoassay (Wingfield et al., 1992) using anticorticosterone antiserum (code B3-163, Esoterix Inc. Endocrinology, CA) and [1,2,6,7-3H]-corticosterone label (Amersham, U.K.). Samples were assayed in one assay and individually corrected for extraction efficiency, with a mean extraction efficiency of 75.6%. The assay was run with 50% binding at 2.11 ng/ml, and the detection limit (for 7.3 μl aliquots of extracted plasma) was 0.4 ng/ml. For testing associations with coloration, we used measurements of baseline and stress-induced CORT, and carotenoids, from samples collected at the beginning of the experimental period (week 0; $CORT_{PRE}$) and at the end of the experimental period (week 12; $CORT_{POST}$).

Carotenoid levels were determined using high-performance liquid chromatography (HPLC) as described in Knott et al. (2010) and, as with CORT, we compared levels in week 0 and week 12 samples, and the change in carotenoid levels over the 12 weeks, to coloration. As previously reported (Knott et al., 2010) carotenoid supplementation significantly increased circulating carotenoid levels compared to food restricted and control treatments (Kruskal-Wallis test, week 0: $H = 2.665$, $df = 2$, $P = 0.264$; week 12: $H = 9.707$, $df = 2$, $P = 0.008$). Heterophil/lymphocyte (H/L) ratio was determined by creating a blood smear on a glass slide with one drop of blood from each bird. Blood smears were air dried and stained using 1 ml of Leishman's stain, prior to visualisation at x100 magnification under oil immersion, within two months of collection. For each slide, 100 leukocytes were counted and classified according to cell type: heterophils (H), lymphocytes (L), basophils, eosinophils, and monocytes; and H/L calculated following Gross and Siegel (1983), blind to the identity of the bird. The H/L ratio has been demonstrated to be a useful measure of chronic physiological stress in birds, where higher H/L ratios reflect a higher level of stress (Davis et al., 2008; Gross and Siegel, 1983; Jones et al., 1988). However, H/L ratio may not always be correlated with glucocorticoid levels or body weight changes (Krams et al., 2012; Müller et al., 2011).

Phytohaemagglutinin (PHA) skin tests were carried out 43 days after the third and final blood sampling at week 12, to avoid confounding corticosterone and carotenoid data. Each bird was injected with the mitogen PHA-P (Sigma, St. Louis, MO) intradermally into each wing web, receiving a 0.1 ml of a suspension of 10 mg PHA-P in 2 ml phosphate buffer saline (1xPBS) per wing (Lochmiller et al., 1993). Digital calipers were used to measure the wing webs before injection (as a control measurement), and at 24 h after injection (to the nearest 0.01 mm), to measure the swelling in response to the mitogen, and the mean swelling of the two wings used for analysis (Martin et al., 2006; Smits et al., 2002).

2.4. Plumage reflectance

We quantified plumage reflectance using protocols similar to earlier studies (e.g. Bennett et al., 1997; Kemp et al., 2015; Pearn et al., 2003) utilising a USB2000 spectrometer and PX-2 xenon light source (Ocean Optics, Dunedin, USA). Reflectance relative to a 99% diffuse white standard was measured through a bifurcated fibre optic (R400-7-UV/VIS, Ocean Optics) held perpendicular to the plumage, with a black terminal sheath over the end of the cable to standardize the illumination area (1.8 mm diameter) and exclude ambient light. We recorded reflectance (300–700 nm) for three psittacofulvin-based plumage patches and three structurally-based patches for each individual. The three pigmentary patches were: 1) the forehead and crown ("crown"), 2) the

ventral surface of the body ("front"), and 3) the rump. The three structural patches measured were: 1) the blue cheek patch on either side of the head ("cheek"), the greater coverts on the right wing ("epaulette"), and 3) the upper side of the outermost rectrix ("tail"). Pigmentary patches were identified by their orange to red coloration (Berg and Bennett, 2010; McGraw and Nogare, 2005), while structural patches were identified by their blue appearance, supplemented by two-dimensional Fourier analysis of transmission electron micrographs of feathers from a sub-sample of birds (Berg and Bennett, 2010) using the methods of Prum et al. (1999). Four replicated measurements were taken of each patch (removing the probe between each measurement and placing it back in a different position). Plumage regions are illustrated in Proctor and Lynch (1993). In the case of the crown, front and tail patches, the four replicates comprised a transect of approximately equally spaced locations along the centre of the patch (or outer vane in the case of the tail), in an anterior-posterior direction, while in the case of the other patches replicate positions were chosen haphazardly from within the patch. All reflectance measurements were made by one person to avoid observer biases.

We calculated three colour variables to summarise reflectance for each of the six plumage patches, as follows. For both pigmentary (long wavelength dominated) and structural (UV-blue dominated) patches, we calculated mean reflectance in the range 300–700 nm (henceforth termed "luminance"). This provided an achromatic measure of reflectance. Additionally, for pigmentary patches we calculated "red chroma" as the ratio of reflectance in the range 575–700 nm compared to the range 300–700 nm (e.g. Siefferman and Hill, 2005), and red "hue" as the wavelength at maximum positive slope in the range 400–700 nm (Keyser and Hill, 1999). For structural patches, we calculated "UV chroma" as the ratio of reflectance in the range 300–400 nm compared to the range 300–700 nm (e.g. Siefferman and Hill, 2005; Smiseth et al., 2001), "blue hue_{peak}" as the wavelength at maximum reflectance in the range 350–450 nm (Siefferman and Hill, 2005; Smiseth et al., 2001), and "blue hue_{slope}" as the wavelength at maximum (negative) slope in the range 350–550 nm (Smiseth et al., 2001). These variables (reviewed in Montgomerie (2006)) were chosen as simple variables that capture the main variation in the typical reflectance curves of pigmentary and structural patches while also making no assumptions about the perception and processing of the optical signals (Kemp et al., 2015). Hue indicates where slope of the spectrum is greatest (Endler, 1990), and the two indices of hue for structural patches, blue hue_{peak} and blue hue_{slope}, were particularly strongly correlated within patches ($0.734 < r_s < 0.894$ for males, $0.748 < r_s < 0.803$ for females). Therefore, we included only blue hue_{slope} (hereafter "blue hue") in further analyses; this variable was chosen to maximize comparability with red hue, which was similarly calculated from the wavelength at maximum slope. To determine the change in coloration during the experiment we subtracted the values for the colour variables for each spectrum at week 0 (commencement of the dietary manipulation) from the same variables measured after mean 26.7 weeks (+3.5 SD, range 20.7–31.7) on the dietary manipulation, at which time the birds were sacrificed as part of another study (13 August to 12 November) (Knott et al., 2010). We then calculated, for each colour variable, the mean change of the four replicate spectra for each plumage patch to use in further analyses. Colour measurements for the three birds within each block were performed in random order within 3–9 days of each other, to avoid confounding treatment effects on coloration with the time receiving dietary manipulation.

2.5. Statistical analyses

To analyse the effects of diet treatment on CORT, we used linear mixed models (LMM). These models incorporated a random intercept representing bird ID to account for repeated measures for birds, and fixed predictors for diet treatment, sampling round (week 0 or 12), and blood sampling time (baseline or stress-induced). To test whether CORT predicted plumage reflectance, we used a MANOVA for each plumage patch, with the change over the 12 week experiment in the three colour variables described above as dependent variables. We used separate models to test the effects of baseline CORT, stress-induced CORT, and change in CORT to avoid multicollinearity. Change in CORT was calculated as CORT measured at the commencement of the experiment (week 0; $CORT_{PRE}$) subtracted from CORT measured at the end of the experiment (week 12; $CORT_{POST}$).

Statistical analyses were conducted in SPSS 25 (SPSS Corp., Armonk, NY, USA). Kolmolgorov-Smirnoff tests and visual examination of residual plots were used to determine whether data adhered to the assumptions of parametric tests, and if necessary non-parametric tests were used. Means and statistical estimates are given ± standard error (SE), unless otherwise stated. To provide an indication of how CORT was associated with not only the linear combination of all colour variables, and also with the three colour variables separately, significant results of both multivariate and univariate tests are reported when $\alpha < 0.05$. For mixed models, denominator degrees of freedom are round to the whole numbers. One bird died during the study (a control) and was excluded from the analyses, and because some CORT measurements were not available for all birds sample sizes varied slightly between some analyses.

3. Results

3.1. Dietary and temporal changes in CORT

Diet treatment (LMM: $F_{2, 13} = 1.844$, $P = 0.197$) and sampling round ($F_{2, 80} = 0.805$, $P = 0.451$; treatment × bleed round interaction $F_{4,701} = 70.332$, $P < 0.704$) did not predict CORT levels overall (Fig. 1). As expected, stress-induced CORT levels were significantly higher than baseline CORT levels ($F_{1, 81} = 23.561$, $P < 0.001$), with no influence of diet treatment (interaction $P = 0.309$). However, stress-induced CORT levels were significantly higher than baseline CORT levels only at the start of the experiment (paired *t*-test, week 0: $t = -6.466$, $df = 17$, $P < 0.001$) and during the experiment (week 4: $t = -3.552$, $df = 14$, $P = 0.003$), but not at the end of the experiment (week 12: $t = -1.900$, $df = 10$, $P = 0.087$; Fig. 1).

Baseline and stress-induced $CORT_{PRE}$ were positively correlated ($r_S = 0.521$, $n = 18$, $P = 0.027$), but no other measures of baseline or stress-induced CORT were correlated during the experiment ($-0.442 > r_S < 0.342$, $P > 0.113$). Change (week 12 minus week 0) in baseline and stress-induced CORT levels were positively correlated ($r_S = 0.645$, $n = 11$, $P = 0.032$).

For the subsample of three birds that were blood sampled over the course of a longer handling/restraint period, at four time periods (< 3 min, 10 min, 30 min and 50 min after capture), CORT increased with time since capture (Fig. S2).

3.2. CORT and plumage reflectance

Stress-induced $CORT_{PRE}$ (measured at the start of the experiment, week 0) was related to colour variables for two pigmentary patches (Fig. S3). For the crown, stress-induced $CORT_{PRE}$ was positively related to luminance ($r^2 = 0.32$, $F_1 = 5.793$, $P = 0.033$) and negatively related to chroma ($r^2 = 0.36$, $F_1 = 6.816$, $P = 0.023$) and hue ($r^2 = 0.44$, $F_1 = 9.567$, $P = 0.009$; multivariate test: Wilks' Lambda = 0.510, $F_{3, 10} = 3.204$, $P = 0.071$). For the front, it was negatively related to chroma ($r^2 = 0.34$, $F_1 = 7.810$, $P = 0.014$) and hue ($r^2 = 0.27$, $F_1 = 5.575$, $P = 0.032$; multivariate test: Wilks' Lambda = 0.624, $F_{3, 13} = 3.204$, $P = 0.096$). No structural patches were predicted by stress-induced $CORT_{PRE}$ (all tests $P > 0.05$; Fig. S3). Baseline $CORT_{PRE}$ or baseline $CORT_{POST}$ did not predict coloration for any patch (all tests $P > 0.05$), nor did stress-induced $CORT_{POST}$ (measured at the end of the experiment, week 12; all tests $P > 0.05$; Fig. S4).

Change in stress-induced CORT over the 12 week experimental

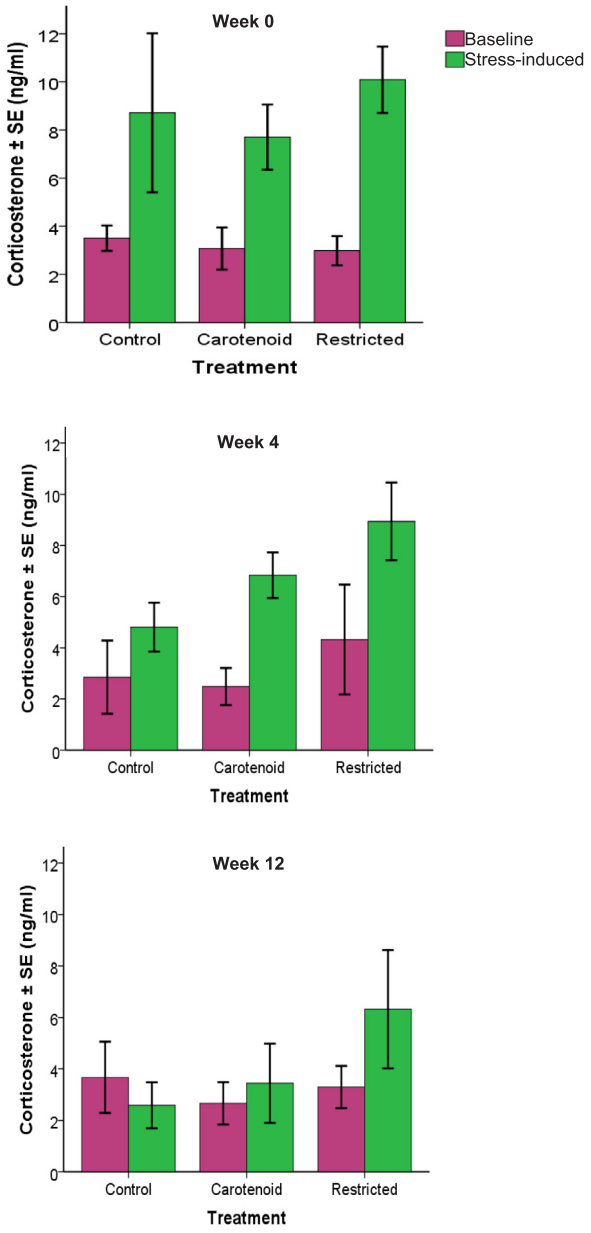

Fig. 1. Baseline (< 3 min after capture) and stress-induced (10 min after capture) levels of CORT (mean ± standard error, ng/ml) for crimson rosellas (*Platycercus elegans*) undergoing control (n = 6), carotenoid supplementation (n = 7) and food restriction (n = 7) dietary treatments. Results are shown for (a) week 0, (b) week 4, and (c) week 12 of the dietary manipulation period. Stress-induced CORT levels were significantly higher than baseline CORT levels at week 0 and week 4, but not at week 12, while sampling week and dietary treatment were not significantly related to CORT levels (see statistics provided in text §3.1).

period predicted the change in colour variables for two structural plumage patches (cheek and epaulette) and one pigmentary patch (crown) (Fig. S5). Among structural patches, change in stress-induced CORT predicted cheek coloration according to the multivariate test (Wilks' = 0.421, $F_{3,\ 9} = 4.128$, P = 0.043) and univariate tests indicated that UV chroma was positively related to change in stress-induced CORT ($r^2 = 0.32$, $F_1 = 5.264$, P = 0.042), but that luminance and hue were not (P > 0.277). Similarly for the epaulette, univariate tests also suggested that UV chroma ($r^2 = 0.31$, $F_1 = 4.835$, P = 0.050), but not luminance or hue (P > 0.167), was positively related to change in stress-induced CORT, however the multivariate test was not significant (Wilks' = 0.595, $F_{3,\ 9} = 2.038$, P = 0.179). For the tail, no colour variables were related to change in stress-induced CORT (univariate tests: P > 0.408, multivariate test: Wilks' = 0.840, $F_{3,\ 9} = 0.570$, P = 0.649).

Among pigmentary patches, univariate tests indicated that change in stress-induced CORT was related to all three colour variables for the crown: negatively with luminance ($r^2 = 0.44$, $F_1 = 8.714$, P = 0.013), and positively with red chroma ($r^2 = 0.47$, $F_1 = 9.846$, P = 0.009) and hue ($r^2 = 0.51$, $F_1 = 11.499$, P = 0.006), however the multivariate test was marginally not significant (Wilks' Lambda = 0.470, $F_{3,\ 9} = 3.382$, P = 0.068). For the other two pigmentary patches (front and rump), neither univariate (P > 0.149), nor multivariate tests (P > 0.519) indicated a significant association with change in stress-induced CORT.

Changes in baseline CORT did not predict change in coloration for any plumage patches according to either univariate tests (structural patches: P > 0.090; pigmentary patches: P > 0.074) or multivariate tests (structural patches: P > 0.312; pigmentary patches: P > 0.126).

3.3. Carotenoids and plumage reflectance

Across all plumage patches and colour variables, we found only one correlation between a colour variable and circulating carotenoid levels measured at week 0, week 12, or as change in carotenoid levels over the experiment: change in luminance of the crown was positively correlated with carotenoid level in week 12 ($r_P = 0.621$, n = 12, P = 0.031). Carotenoid levels were not correlated with any measure of CORT during the experiment (-0.222 < rs < 0.476, P > 0.086). Carotenoid levels measured in week 0 and 4 were positively correlated (r = 0.696, n = 20, P = 0.001), but levels at the end of the experiment (week 12) were not significantly correlated with week 0 (r = 0.402, n = 20, P = 0.079) or week 4 (r = 0.388, n = 20, P = 0.091).

3.4. Changes in heterophil/lymphocyte ratio and PHA-induced immune response

Treatment significantly predicted heterophil/lymphocyte ratio (Kruskal-Wallis: H = 7.908, df = 2, P = 0.019), with carotenoid (U = 41.000, P = 0.002), but not food restricted birds (U = 33.000, P = 0.101), having significantly higher ratios than control birds (Fig. S6a). There was no effect of treatment on PHA-induced immune response (H = 1.780, df = 2, P = 0.411; Fig. S6b). We found no correlations between either heterophil/lymphocyte ratio or PHA response and any measure of CORT or carotenoids ($-0.364 < r_S < 0.316$, P > 0.182).

4. Discussion

In this study, we tested, for the first time in a parrot species, whether physiological stress levels resulting from dietary restriction and/or carotenoid supplementation were related to the change in coloration during plumage moult. In doing so we sought to test the mechanisms determining the expression of plumage coloration in parrots, a little studied group. We studied crimson rosellas, a species for which we have found that structural coloration is more strongly associated with body condition that psittacifulvin based coloration (unpublished results). Our results provided evidence that variation in stress-induced CORT predicted pigmentary, and to a lesser extent structural, coloration in this species depending on the plumage patch, that the stress-induced CORT response following handling changed over the course of the study, and that leucocyte counts were influenced by carotenoid intake. As expected, there was limited evidence that either type of coloration was associated with carotenoid levels. These findings are discussed in turn below.

4.1. Diet treatment and stress

As previously reported, our diet manipulation resulted in changes in predicted directions in circulating carotenoid levels and body weight (see Fig. S1; Knott et al., 2010). Here, we showed diet manipulation also affected heterophil/lymphocyte ratio (carotenoid supplemented birds were higher) but, unexpectedly, not PHA response or CORT. Although our sampling regime did not measure peak CORT, but an elevated response only, our CORT results suggested a pronounced attenuation of the stress response (10 min post capture) during the progression of the experiment, with acutely elevated (stress-induced CORT), but not chronic (baseline CORT) stress levels dropping substantially across the weeks of sampling (Fig. 1), during which birds were handled twice weekly. Although it should be noted that the endocrine response to chronic stress can be extremely variable (Dickens and Romero, 2013), these data are consistent with a reduced responsiveness of the HPA axis due to the chronic stress of long term captivity and repeated handling (Blanchard et al., 1995; Rich and Romero, 2005). The elevated CORT levels we measured did not to reflect 'peak' CORT production in at least the three individuals sampled over a longer period, the inter-individual variation appears similar at 10 min and 50 min post capture (Fig. S2). However, it is plausible that blood sampling at longer time frames post-capture would have revealed different relationships between plumage coloration and peak CORT levels. The associations between the expression of structural and pigmentary colour patches and changes in HPA responsiveness across our experiment suggests that there may be mechanistic links between functional control of the stress response and both mechanisms of colour production, allow colour signals in parrots to act as honest indicators of recent experience.

Our second indicator of chronic stress, the H/L-ratio (Davis et al., 2008; Gross and Siegel, 1983; Müller et al., 2011) suggested chronic stress may have been higher in the carotenoid treated group. The reasons for this effect remain unclear, and as predicted for parrots we found little indication of associations between dietary carotenoids and plumage colour production (discussed below). Although H/L-ratio is widely used and well supported measure of chronic stress (Davis et al., 2008; Gross and Siegel, 1983; Müller et al., 2011), it is not always correlated with glucocorticoid levels or body condition and may instead covary with humoral immune response (Krams et al., 2012; Müller et al., 2011). Moreover, leucocyte counts may also respond to short term changes in stress and H/L ratio in birds may rise within 0.5–18 hr after exposure to a stressor (Davis, 2005; Gross, 1990; Mills et al., 1993), although in our study blood was sampled < 10 min after capture so the H/L ratios were unlikely to be affected by handling stress. Finally, in contrast to studies in other taxa, we found no correlations between immune function (PHA response) and either CORT or carotenoid levels, and no difference in PHA response across treatments. Further studies are required to confirm whether immune function has similar relationships to stress and dietary carotenoids in parrots as known in in other taxa.

4.2. Corticosterone and coloration

The plumage patches most clearly associated with CORT were the cheek and epaulette (two of the three structural patches) and the front and the crown (two of the three pigmentary patches). For the structural patches of cheek and epaulette, change in stress-induced CORT was associated with an increase in UV chroma during the study. Crown colour was related to both stress-induced $CORT_{PRE}$ and the change in stress-induced CORT, based on significant univariate tests for all three colour variables (but the multivariate tests were marginally non-significant for both CORT variables). Front colour was related only to stress-induced $CORT_{PRE}$ as shown by significant univariate tests for two colour variables (again accompanied by a marginally non-significant multivariate). Note however that only effects on colour variables for the two pigmentary patches (crown and front) would withstand correction for at least three multiple comparisons (accounting for the three colour variables). Although change in stress-induced CORT was related to colour variables for three patches, stress-induced $CORT_{POST}$ itself was not significantly related to any colour variables for any patch. Moreover, we also only found effects involving stress-induced CORT and not baseline CORT. The reasons for this are unknown, but one hypothesis is that the level of CORT reached in response to an acute stressor, such as capture and handling, plays a more important role in colour expression than baseline levels of CORT. It is important to note that our measure of stress-induced CORT was only an elevated CORT response (10 min post capture), and not the peak CORT response, which may have some bearing on the interpretation of our results. It is also important to recognise that whilst our results are consistent with the interpretation that CORT plays a role in mediating colour expression, it is possible that this is an indirect consequence of our dietary manipulations. Further experimental study is needed to eliminate this possibility, for example using a non-dietary stressor or direct administration of CORT. Closer examination of the biological relevance of CORT for colour expression over the entire stress response is also needed.

Numerous studies investigating single ornaments of different bird and reptile species have found that CORT levels were associated with the expression of different types of coloration (Calisi and Hews, 2007; Fitze et al., 2009; Grindstaff et al., 2012; Loiseau et al., 2008; Martínez-Padilla et al., 2013; Roulin et al., 2008). This has led to the suggestion that stress hormones, which may negatively affect condition at least in situations of long-term stress (Cote et al., 2010a,b, Moore and Miller, 1984; Tokarz, 1987), may have a general effect on the condition dependence of colour ornaments, even for multiple ornaments based on different colour mechanisms in the same individual (San-Jose and Fitze, 2013). The roles of glucocorticoid hormones in mediating plumage coloration have been demonstrated in a number of species, particularly for pigmentary (melanin- and carotenoid-based) colour traits including carotenoid-based yellow-red plumage (e.g. Grunst et al., 2015; Lendvai et al., 2013).

As with condition dependence generally, less evidence is available for a relationship between CORT and structural plumage coloration, but such associations have been found in at least two species. Henderson et al. (2013) observed a negative correlation between UV colour and baseline CORT in female blue tits (*Cyanistes caeruleus*), but this was not the case in eastern bluebirds (*Sialia sialis*), where a positive correlation existed between CORT and UV hue and saturation (Grindstaff et al., 2012). Studies of pigmentary coloration have similarly yielded conflicting results. Some studies have reported negative relationships between CORT concentrations and pigmentation, suggesting that CORT may suppress the deposition of pigments into the integument in favour of other functions (such as antioxidant activity or immune function), or that CORT is correlated with poor condition and an inability to produce concentrated pigmentation (Martínez-Padilla et al., 2013; Mougeot et al., 2010). Conversely, findings of positive relationships between CORT and pigmentation suggest that CORT may enhance the uptake, processing, or deposition of pigments (Cote et al., 2010a,b; Fairhurst et al., 2014; Lendvai et al., 2013). The lack of consistent patterns may make sense if traits based on different colour producing mechanisms, such as carotenoid or melanin pigments, or integument structure, serve non-equivalent functions (Grunst et al., 2015; Moller and Pomiankowski, 1993), or if CORT represents not simply an index of condition or quality but instead mediates complex mechanisms of colour expression (Lendvai et al., 2013).

Tests in taxa using novel colour mechanisms may provide useful further insights, and parrots make an interesting system for such studies. Parrots generate plumage coloration in part using psittacofulvin pigments. These unique pigments, responsible for the long wavelength (yellow, orange and red) coloration in parrot plumage, are thought to be a distinguishing evolutionary novelty of parrots found nowhere else in nature (McGraw and Nogare, 2005; Stradi et al., 2001). Our results support an association between CORT and colour produced by both

structural and pigmentary mechanisms, although not all plumage patches seemed to be affected the same. These findings concur with San-Jose and Fitze (2013) who, studying common lizards (*Lacerta vivipara*), found that experimentally manipulated CORT was related to both melanin and carotenoid coloration in the same individuals, but only for ventral (sexually selected) and not dorsal (not sexually selected) coloration. It is possible that the plumage patches where we found effects are more strongly condition dependent and/or sexually selected. Another possibility is that the timing, duration or completion of moult varied between individuals or diet treatment, and that this contributed to some of the associations that we found. Although we cannot rule this out in the current study, we think it is likely that subjects moulted during the diet manipulation period because: (i) our final colour measurements we made from late summer to late autumn (20.7 to 31.7 weeks after diet manipulation commenced, depending in the experimental block), so occurred after the main natural moulting period reported for *P. elegans*, and a sufficient time after dietary manipulation commenced for moult to be completed (Higgins, 1999); (ii) all birds used in this experiment were observed to be moulting throughout the manipulation period, and over the same period the previous year when housed in the same way; and (iii) we observed no differences in feather growth rates or the timing of moult in relation to diet treatment during our study (unpublished results), and in studies of wild populations no differences in timing or sequence of moults have been reported across six subspecies of *P. elegans* occupying diverse environments in south-eastern Australia (Higgins, 1999). Future studies using experimental CORT administration (Jenni-Eiermann et al., 2014) or a non-dietary stressor, perhaps in combination with experimental manipulation of condition and more detailed quantitative measures of moult, will be invaluable in further elucidating the patterns we have reported here.

4.3. Carotenoids and coloration

In many species, carotenoids may be in limited dietary supply and act as antioxidants and immunostimulants, as well as pigments underlying sexual coloration in many species (e.g. Hill, 2006; Hill, 2011; McGraw, 2005; McGraw and Ardia, 2003; Schantz et al., 1999). Thus, carotenoid pigmentation often has condition-dependent expression and so readily serves as a sexual ornament, and it is well established that carotenoid intake mediates the expression of carotenoid-based colour traits in many taxa, including most avian families (Hill, 2006; Hill, 2011). This effect is thought to arise due to a direct mechanistic link between the amount of carotenoids consumed and the amount available for deposition in the integument, and thereby to provide an honest indicator signal (Hill, 2006; Hill, 2011; Olson and Owens, 1998).

It is expected that this effect would be less likely in Psittaciformes, because members of this order produce plumage coloration using unique psittacifulvin pigments rather than carotenoids, along with non-pigmentary structural coloration. Psittacofulvins are thought to be produced endogenously at the feather follicle and not derived from dietary sources (Mcgraw, 2006; McGraw and Nogare, 2004; McGraw and Nogare, 2005). However, parrots do accumulate dietary carotenoids in the body (Knott et al., 2010; McGraw and Nogare, 2004) and it is plausible that an indirect link between carotenoid levels and plumage coloration in parrots may still occur if parrot coloration is related to general condition of the bird or immune function, since carotenoids are important immune-modulators and part of the antioxidant system (reviewed in Bendich, 1993; Edge et al., 1997; Hill, 2011). We tested for the first time whether circulating carotenoid levels predicted coloration in parrots, and found that such associations were largely absent: the exception was that change in luminance of the crown was significantly correlated with carotenoid levels in week 12. Our results therefore suggest that crown coloration may be associated both with CORT and carotenoid accumulation in some way, but such a limited effect requires confirmation in further studies. Overall, our results support the hypothesis that Psittaciforme pigmentation, unlike the carotenoid pigmentation used for yellow/orange/red coloration in most avian taxa, is insensitive to dietary carotenoid intake (Berg and Bennett, 2010).

4.4. Conclusions

Taken together, our findings indicate for the first time that corticosterone may be linked to the expression of psittacifulvin-based plumage coloration in parrots, and thus to animal coloration more broadly that previously thought. The results also add to evidence that not only pigment-based but also structural coloration may be related to glucocorticoid levels, even in the same individual and species. Our study also provides novel experimental support for the long-held but hitherto untested assumption that yellow-red plumage pigmentation in Psittaciformes is insensitive to dietary carotenoids, unlike many other avian taxa. Finally, our findings provide new information on the physiology and stress response of Psittaciformes, which is valuable as they are one of the most highly threatened, yet little studied, avian orders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.014.

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