Electronic supplementary material: Methods

Experimental design

Detailed information on the composition of hihi nestling diet is lacking, and so the doses of the nutritional treatment were based on the manufacturer’s recommendations. The manufacturers of Wombaroo Lorikeet & Honeyeater Food recommend that wild honeyeaters be fed a daily maximum of 5 mL of a 30% by mass Wombaroo solution. Given the range of honeyeater body mass (8 – 200 g; [1]), and the nutritional composition of Wombaroo (140 g kg\(^{-1}\) protein, 60 g kg\(^{-1}\) fat, 6 g kg\(^{-1}\) fibre), this represents a daily intake of 0.1 - 2.6 % protein, 0.05 - 1.1 % fat, and 0.005 - 0.1 % fibre, expressed as a percentage of body mass. In our experiment, we used a fixed concentration of Wombaroo (0.3 g mL\(^{-1}\)) and increased volume with age to achieve protein, fat and fibre amounts within the recommended intake (Table S1).

We followed the recommendations of [2] to calculate daily carotenoid doses. Mean hihi fledgling body mass is 36.15 g (± 0.46, n = 192) and mean plasma carotenoid concentration is 6.0 µg mL\(^{-1}\) [3]. Given that blood volume represents about 10% of nestling body mass [4], total quantity of circulating carotenoids in fledgling hihi plasma can be estimated as 21.69 µg. Assuming the efficiency of carotenoid absorption in birds is about 20% of the used dose [5], we aimed to supplement 100 µg (i.e. five times the total quantity of circulating carotenoids) per feeding. Carotenoids were supplemented at a final concentration of 100 µg mL\(^{-1}\) in volumes of 0.2 mL to 3.0 mL (Table S1), such that amount supplemented ranged from 20 µg at 4 days old to 300 µg at 20 days old. This allows for the growth of nestlings over the 17-day
period of supplementation, and is equivalent to receiving a daily carotenoid supplement of 80 µg.

Table S1: the volume of Wombaroo, and the resulting amount of protein, fat and fibre, expressed in absolute values and as a percentage of body mass, supplemented at each age

<table>
<thead>
<tr>
<th>Age</th>
<th>Vol (mL)</th>
<th>Protein (g)</th>
<th>Fat (g)</th>
<th>Fibre (g)</th>
<th>Mean body mass (g)</th>
<th>Protein (as % of body mass)</th>
<th>Fat (as % of body mass)</th>
<th>Fibre (as % of body mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.0084</td>
<td>0.0036</td>
<td>0.00036</td>
<td>5.79</td>
<td>0.15</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>6</td>
<td>0.3</td>
<td>0.0126</td>
<td>0.0054</td>
<td>0.00054</td>
<td>9.39</td>
<td>0.13</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>0.021</td>
<td>0.009</td>
<td>0.0009</td>
<td>14.24</td>
<td>0.15</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>0.042</td>
<td>0.018</td>
<td>0.0018</td>
<td>19.62</td>
<td>0.21</td>
<td>0.09</td>
<td>0.009</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
<td>0.063</td>
<td>0.027</td>
<td>0.0027</td>
<td>24.28</td>
<td>0.26</td>
<td>0.11</td>
<td>0.011</td>
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<tr>
<td>14</td>
<td>2.0</td>
<td>0.084</td>
<td>0.036</td>
<td>0.0036</td>
<td>28.15</td>
<td>0.30</td>
<td>0.13</td>
<td>0.013</td>
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<td>16</td>
<td>2.4</td>
<td>0.1008</td>
<td>0.0432</td>
<td>0.00432</td>
<td>30.59</td>
<td>0.33</td>
<td>0.14</td>
<td>0.014</td>
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<tr>
<td>18</td>
<td>2.8</td>
<td>0.1176</td>
<td>0.0504</td>
<td>0.00504</td>
<td>32.89</td>
<td>0.36</td>
<td>0.15</td>
<td>0.015</td>
</tr>
<tr>
<td>20</td>
<td>3.0</td>
<td>0.126</td>
<td>0.054</td>
<td>0.0054</td>
<td>34.09</td>
<td>0.37</td>
<td>0.16</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Plasma carotenoid concentration

20 µL plasma was homogenised with 20 µL NaCl 5% and 40 µL ethanol, and carotenoids were twice extracted by homogenization with 500 µL hexane. Hexane
extracts were pooled and evaporated at 70 °C under nitrogen flow, and the residue
was dissolved in 50 µL dichloromethane and 50 µL methanol. Total carotenoids were
detected with a Spherisorb type S5NH2 reverse-phase column (25 cm x 4.6 mm,
Phase Separation Ltd., Clwyd, UK) with a mobile phase of methanol (97:3) at a flow
rate of 1.5 mL min\(^{-1}\), using UV detection by absorbance at 445 nm.

*Plumage colour analysis*

Given the differences between avian and human vision, plumage colour analysis
should take into account the avian visual system [6–9]. Therefore, reflectance spectra
were analysed using models in tetrahedral colour space to extract hue, saturation and
luminance variables for each colour patch [10–12]. Hue, the colour type (e.g. blue
versus red), and saturation, the amount of colour compared to white light, can be used
to describe the colour of a patch. Luminance captures the achromatic component of
the signal and describes the perceived lightness of a patch. We first calculated photon
catch values for the four single cones, used in colour vision, and the double cones,
used in luminance vision, based on the reflectance spectra and measures of irradiance
[12]. Models were run using standard ‘d65’ (full light) and ‘forest shade’ irradiance
spectra. In addition, since the spectral sensitivities of hihi are not known, we
calculated photon catches using spectral sensitivities from a species with an
ultraviolet shifted shortwave sensitive cone type (UVS) (blue tit; *Cyanistes caeruleus*)
[13] and a species with a violet (VS) system (peafowl; *Pavo cristatus*) [14]. Results
did not differ significantly when using different irradiance levels and spectral
sensitivities, therefore only values generated using ‘d65’ irradiance levels and with
blue tit spectral sensitivities are presented. Following this, our measure of luminance
was simply the double cone photon catch values. To calculate saturation, we plotted the standardized single cone catch data for each individual (using relative cone catches to remove variations in absolute brightness) in avian tetrahedral colour space [12] and calculated the distance from the centre of the colour space. Larger values indicate greater saturation. To calculate hue, we adopted an approach similar to [15] and [16]. We used ratios based on photon catch outputs that are broadly inspired by the way that opponent colour channels work, based on performing a principal component analysis (PCA) on a covariance matrix of the standardized single cone data (see [17]). The PCA enabled us to encode colour in a biologically meaningful way [15,16]. The first principal component (PC) explained 97 percent of the variation in yellow plumage colour, with the colour channel being: (LW+MW)/(SW+UV), where LW, MW, SW and UV indicate longwave, mediumwave, shortwave and ultraviolet cone catches, respectively. The same colour channel explained 85 and 84 percent of the variance for the black and white patches respectively.

Data analysis

Variation in plasma carotenoid concentration was explored by fitting a linear mixed effects model using the lme function in R’s nlme package. This function calculates degrees of freedom in such a way that the random effect needn’t be explicitly nested in the nutritional treatment, as some other statistical programs require. A full description of how this function calculates df can be found in [18]. Briefly, df for a level 1 variable (which takes a different value for all observations within the levels of the random effect e.g. carotenoid treatment) are calculated as the number of level 1 observations minus the number of level 2 clusters minus the number of level 2 fixed
effects. The df for a level 2 variable (which takes the same value for all observations within the levels of the random effect e.g. nutritional treatment) are calculated as the number of level 2 clusters minus the number of level 2 variables minus 1 for the intercept. With 188 individuals and 73 nests in the case of carotenoid concentration, this gives 73-3-1 = 69 df for nutritional treatment (a level 2 variable) and 188-73-2 = 113 df for carotenoid treatment (a level 1 variable) (as reported in the results).

When investigating the effects of treatments and covariates on colour variables, a re-sampling approach was taken to control for the minority of cases where siblings from the same brood were measured. A single nestling was randomly drawn from each nest to generate a sample dataset equal in size to the number of nests (e.g. yellow post-moult models had one nestling drawn from each of the 35 nests that were represented). 1000 sample datasets were generated (with a random nestling being sampled from each nest each time) and linear models were fitted to each sample dataset, thus generating a model distribution for each of the effects. Mean effect sizes, standard errors, and t-values from this re-sampled distribution are reported, along with the 95% confidence intervals around the mean t-value and the percentage of re-sampled p-values that fall below 0.05.

In cases where residual plots of plumage colour models revealed substantial heterogeneity, linear models were fitted using generalised least squares with a specified variance structure (gls function in R). The post-moult yellow plumage saturation model allowed variance to differ by moult score (using varIdent function in R); the pre-breeding black plumage saturation model allowed variance to differ by nutritional treatment (using varIdent); the post-moult white plumage luminance model
allowed variance to differ by hatch date (using varExp); the pre-breeding white plumage saturation model allowed variance to differ by hatch date (using varExp); and the pre-breeding white ear-tuft length model allowed variance to differ by nutritional treatment (using varIdent).

References


