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ORIGINAL PAPER

Soil and preen waxes influence the expression of carotenoid-based plumage coloration

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Abstract The signaling function of carotenoid-based plumage is mainly determined by the concentration of pigments in feathers. For this reason, most studies of the proximate control of coloration focus on processes during and preceding moult. In great tits *Parus major*, past research demonstrates that carotenoid-based plumage coloration honestly indicates male quality and, thus, may be a sexually selected signal. In this study, we investigate how dirt and preen oil influence the coloration of carotenoid-based feathers in the great tit. We collected six feathers from each individual bird; three feathers served as controls while the remaining three feathers were washed with a chloroform/methanol mixture to remove soil and preen waxes. We assessed plumage coloration using digital photography. This cleaning procedure slightly enhanced ornamentation; the experimentally cleaned feathers expressed hues shifted towards shorter wavelengths and expressed brighter overall coloration than control feathers. This is the first experimental study conducted on wild birds demonstrating that, in addition to pigment concentration, the presence of preen waxes and soils on feathers may contribute to variation in coloration.

Keywords Plumage color · Hue · Uropygial gland secretions · Plumage maintenance · Sexual selection

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Introduction

Many bird species exhibit brilliantly colored feathers, beaks, or legs. Regardless of its origin, the intensity of coloration is often closely correlated with individual condition (Griffith and Pryke 2006). For this reason, integument colors are often crucial cues used in mate choice (Hill 2006) and/or social status signaling (Senar 2006). The proximate mechanisms of pigment-based plumage color expression are relatively well studied and include pigment acquisition from the diet, metabolism, and transport in blood stream (Hill 2002).

Although colors of soft tissues (e.g., beak and legs) may change relatively quickly and respond to the health status of their owner (e.g., Faivre et al. 2003), feather coloration is more static. Once coloration is established in the barbules, those colors are separated from any agents present in a blood stream. Thus, it is widely assumed that feather coloration is a stable signal reflecting the body condition of birds at the time during and immediately proceeding moult. There is evidence, however, that feather coloration changes during the breeding season (Örnborg et al. 2002; McGraw and Hill 2004; Figuerola and Senar 2005; Delhey et al. 2006). There are several possible mechanisms that may be responsible for modification of the coloration of fully grown feathers including mechanical abrasion (e.g., Willoughby et al. 2002), bacterial degradation (e.g., Grande et al. 2004), application of preen waxes (Piersma et al. 1999), presence of ectoparasites (e.g., Moreno-Rueda 2005), and soiling (e.g., Zampiga et al. 2004). In virtually all cases, these "post-moult" mechanisms of change in feather coloration are not understood fully and are based on scarce data.

Except rare cases in which birds deliberately soil feathers to enhance camouflage (Montgomerie et al. 2001) or sexual attractiveness (Negro et al. 1999), the effect of

dirt accumulation on feather coloration has received little research attention. Montgomerie (2006) cleaned feathers of three species of museum specimens to remove soil and resins, and using spectrometry, demonstrated an effect on reflectance properties. Zampiga et al. (2004) experimentally demonstrated that contaminated feathers depress UV reflectance in male budgerigar *Melopsittacus undulatus* and cause them to be less attractive to females. Also, the seasonal reduction of UV chroma of the structural plumage coloration of blue tits *Parus cearuleus* is suspected to result from accumulation of soil and (or) wax (Örnborg et al. 2002). The hypothesis that preen wax enhances bird plumage coloration (Piersma et al. 1999) was tested using the red knot *Calidris canutus*; however, no significant change in the reflectance spectra before and after wax (monoesters and diesters) removal was found (Reneerkens and Korsten 2004).

The great tit *P. major* is a small, hole-nesting, passerine with yellow ventral coloration and a distinctive black breast stripe. The yellow plumage of the great tit derives from two carotenoids: lutein and zeaxanthin (Partali et al. 1987). These plumage characteristics are sexually dichromatic; males have larger breast stripes and display yellow coloration with hues that are shifted toward the shorter wavelengths compared to females (Hörak et al. 2001, but see Senar et al. 2003 for the lack of intersexual differences). The hue of yellow breast in great tit is dependent on individual condition in males (Hörak et al. 2001; Senar et al. 2003), suggesting that it may be a target of sexual selection (Hörak et al. 2001).

In this paper, we make the first attempt to experimentally test the effect of naturally deposited soil and preen wax on the carotenoid-based plumage coloration in the wild-living population of passerine species. We discuss the potential effect of feather contamination on fitness and sexual selection of the great tit.

Materials and methods

Field methods

We collected feather samples from 105 yearling males caught in mist nets during spring migration (26 March–11 April 2006) at two ringing posts located on the Polish coast of the Baltic Sea (Bukowo–Kopań, 54°21′N and 16°20′E; Hel, 54°36′N and 18°48′E, for habitat description see Nowakowski 2001). We performed color measurements on six feathers that were plucked using plastic forceps from a standard position on the breast: the midpoint between the upper part of the sternum and the edge of the left wing (followed by Hörak et al. 2001). After plucking, we placed feathers in black plastic boxes and stored the feathers in darkness until color measurements were taken.

Feather cleaning

We collected six feathers from each individual and randomly assigned three feathers to the control group and three feathers to the treatment group. In the treatment group, feathers were cleaned of oil wax and dirt using the following protocol from Poltz and Jacob (1974) to extract preen oil from great tits' uropygial glands. Feathers were immersed in a tight box containing 10 ml of chloroform/methanol mixture (2:1), shaken, and left for ca. 2 min. Next, the feathers were shaken again, removed from the box, and dried. To assess the relative amount of non-soluble soiling and preen wax present on feathers surfaces, we followed the procedure by Sandilands et al. (2004). In short, the method derives an amount of preen waxes from the weight difference between intact and subsequently cleaned feathers (Sandilands et al. 2004). We modified this procedure by shortening the cleaning time and using a specific solvent of an ambient temperature to ensure that cleaning would not disturb feather structure. Moreover, we separate soil from residue removed from feathers. Feathers from additional samples (n=6) were placed in a small flask and rinsed twice with chloroform/methanol mixture. Next, the liquid was poured into another flask through a paper filter. The filter was additionally rinsed with small amount of fresh chloroform/methanol mixture. The chloroform/methanol mixtures from the flask and the filter were evaporated in 30°C. The empty flask and the dry filter were then weighted to the nearest 0.1 mg with electronic balance (WAX 220, RADWAG Radom, Poland). We estimated the amount of preen waxes and soil present on feathers by calculating the difference between obtained masses and the known masses of "clean" flasks and filters, respectively. We assumed that all substances were preen waxes if it dissolved in chloroform/methanol and remained in the flask after evaporating. Similarly, the residue remaining on the paper filter was assumed to be soiling from the feathers' surface.

Color measurements

Feathers from the control and treatment group belonging to the same bird were placed on black plastic cards (3×3 cm) in separate places (left and right side of the card, respectively). In each group, feathers were layered to maximally cover the black card behind them. The black card was covered with self-adhesive white plastic foil (3×3 cm) supplied with two round holes (ø 5 mm) made in standard positions, 5 mm from the sides and 5 mm from the top. In this way, the area of color measurements was confined to 5 mm circles, located in approximately the same feather region in all birds studied.

We measured color using the methods derived from earlier studies of great tits (e.g., Fitze and Richner 2002;

Tschirren et al. 2005). In short, we photographed feathers with a digital camera (Nikon D70s, objective AF-S Nikkor 1:3.5–4.5 G with Soligor extension tube 12). To standardize light conditions, we took all photographs in a tight wooden box (30×30×30 cm) using a ring flash (Nikon Macro Speedlight SB-21) mounted on the distal edge of the lens. We placed the feather cards (two for each exposure) in a standard position with the distance to the front lens fixed to 12 cm. We used identical settings of the camera and flash to ensure that all photographs received standard light exposure. We fixed two standard yellow chips above and below each card to allow calibration of the equipment during analyses. We imported the photographs into the Adobe Photoshop program, which calculates mean hue–saturation–brightness values (HSB). The variation in light exposure, as assessed from the measurements of both yellow reference chips, was small (H_1, 54.2±0.4°; S_1, 34.0±0.6°; B_1, 71.2±1.3%; H_2, 53.1±0.3°; S_2, 33.8±0.5%; B_2, 72.0±1.3%); therefore, no correction of measured color values was required.

To assess repeatability (Lessells and Boag 1987) of color measurements, we photographed 48 feather samples twice. Repeatabilities of all color parameters were significant (H, r=0.999, $F_{1,47}$=9.8, p<0.001; S, r=0.999, $F_{1,47}$=185.9, p<0.001; B, r=0.999, $F_{1,47}$=59.4, p<0.001), indicating that the color measurements were well standardized. This method of measuring color is not sensitive to UV light that is visible for birds (Cuthill et al. 2000) and, in some species, plays a role in mate choice (see Hill 2006 for a review). The yellow breast plumage of great tits shows a reflectance peak within 300–400 nm (Senar and Quesada 2006), but its function has not been studied. However, we assume that this UV peak would not bias our results because the reflectance peaks in the UV and yellow–red spectrum (500–700 nm) are positively correlated (Senar and Quesada 2006), thus, using both measurements would be redundant.

Statistical analysis

HSB values were partially intercorrelated (saturation–hue: r_s=−0.57, p<0.05, and n=210; saturation–brightness, r_s=−0.14, p<0.05, n=210; brightness–hue: r_s=0.12, p<0.07, n=210); thus, we used a principal components analysis (PCA) to condense our plumage measurements (following the methods of Fitze and Richner 2002). This analysis allowed us to reduce the number of variables that we used to describe plumage coloration and to elucidate the co-linearity in our color measures. Only one principal component emerged with an eigenvalue greater than 1.0, and this was used as an overall measure of the plumage coloration (hereafter referred to as PC1). PC1 explained 55% of the variation in plumage coloration (eigenvalue = 1.659; component loadings, hue = 0.88; saturation = −0.88; brightness = 0.33). Thus, higher PC1 scores refer to birds that exhibit reflectance peaks at shorter wavelengths (are more "yellow") and that exhibit less-saturated plumage, while individuals with lower PC1 scores refer to birds that exhibit reflectance peaks in the longer wavelengths (are more "red") and that exhibit more-saturated plumage. We know from other studies of great tits (Hörak et al. 2001; Senar et al. 2003) that males with breast plumage with reflectance peaks shifted toward shorter wavelengths (more "yellow") exhibit better body condition. On the other hand, no relationship was found between body condition and the saturation or brightness of yellow plumage (Senar et al. 2003). Thus, we assumed that more-ornamented males exhibit higher hue scores and, overall, higher PC1 scores.

We tested for normality and equality of variances using Kolmogorov–Smirnov and Levene's tests, respectively. PC1 data conformed to normality but HSB data did not. To investigate the effects of capture locality on variation between overall plumage coloration (PC1), we performed repeated-measures analyses of variance (ANOVAs). We tested whether carotenoid-based coloration differs between localities. Within-subjects factors were tested for variation in coloration caused by the cleaning procedure performed on feathers taken from the same individual. To compare the HSB and PC1 between the cleaned and control feathers taken from the same individuals, we used Wilcoxon's tests for matched pairs and t test for matched pairs, respectively. We investigated the relationship in overall coloration (PC1) between clean and dirty feathers taken from the same birds using Pearson's correlation. All tests were two-tailed with a 0.05 significance level. Means ± SD are given throughout the text.

Results

The average mass of soiling found on feathers (0.610±0.712 mg) was significantly higher than preen wax (0.063±0.0314 mg; Wilcoxon's test, z=2.2, p<0.03, n=6). Mean HSB values for control feathers (H, 54.8±1.6; S, 37.5±4.6; B, 37.4±2.8), were, on average, lower than for cleaned feathers (H, 55.5±1.4; S, 37.8±4.3, B 38.4±2.6). Statistically significant differences, however, were found only for hue (Wilcoxon's test, z=3.5, p<0.001) and brightness (Wilcoxon's test, z=3.2, p=0.001), but not for saturation (Wilcoxon's test, z=−0.8, p=0.41). Similarly, PC1 values obtained for control feathers (−1.15±1.04) were significantly lower than those of cleaned feathers (1.15±0.94; paired t test, t=−2.8, p=0.006, Fig. 1). Overall coloration (PC1) of control and cleaned feathers were significantly correlated (r=0.42, p<0.001, n=105, Fig. 2).

According to repeated-measures ANOVA results, bird coloration did not differ significantly with capture location (Table 1). Variation in coloration caused by the cleaning procedure was significant, but did not vary with locality (Table 1).

Discussion

Our study provides the first experimental evidence that the presence of soil and preen wax influences the coloration of carotenoid-based plumage in wild-living birds. Feathers that were cleaned exhibited coloration with hues shifted further into the shorter wavelengths (indicated by an increase in hue values) and were lighter (greater brightness values) compared to the control feathers. The saturation remained unchanged. Thus, removing soil and preen wax appears to have increased the overall ornamentation of the yellow breast plumage of great tits. It is unlikely that the experimental protocol (chloroform/methanol wash) influenced color by removing pigments from keratin (Kevin McGraw, personal communication; see also McGraw et al. 2005). We are, therefore, confident that the observed results were caused by the removal of soil and waxes.

Unfortunately, we cannot explicitly assess the relative contribution of these two agents (soil and wax) in influencing the change in color; however, earlier studies suggest that soiling is the main factor. No effect of preen wax on plumage coloration was shown (Reneerkens and Korsten 2004) by contrast with soiling which did (Zampiga et al. 2004; Montgomerie 2006). Nevertheless, it is important to point out that, in all studies cited above, only one of the factors (preen wax or soiling) was taken into account. The mechanism of plumage color change due to both dirt or preen waxes is poorly studied. According to studies by Örnborg et al. (2002) on the blue tit, both dirt and fat absorb light mainly at shorter wavelengths, and result in shifts of the reflectance peaks towards longer wavelengths. Also, in one type of preen waxes produced by red knots (diesters), absorbance decreased with increasing wavelength (Reneerkens and Korsten 2004). In this way, our results are consistent with the findings of Örnborg et al. (2002) and Reneerkens and Korsten (2004)—"dirty" feathers of great tits exhibited lower hue values, which are equivalent to longer wavelengths in the reflectance spectra obtained by using of spectrophotometer. On the other hand, Montgomerie (2006) showed that the effect of dirt on plumage reflectance varies strongly according to the type of soiling, initial coloration, and species. For example, resin from nesting trees has different effects on the hue of the yellow plumage of evening grosbeaks *Coccothraustes vespertinus* (no change in plumage coloration) and the red plumage of pine grosbeaks *Pinicola enucleator* (soiling caused a shift toward shorter wavelengths; Montgomerie 2006). In general, changes in coloration reported by Montgomerie (2006) were more pronounced than in our study and were visible to the human eye. However, the species studied by Montgomerie (2006) were especially likely to be exposed to soiling due to their habitat (urban pollutions, resins). Moreover it is important to note that, in museum specimens, part of soiling load could be acquired during specimen storage. Indeed, feather degradation of carotenoid-based coloration in museum specimens is positively related to the length of time the specimen was in storage (McNett and Marchetti 2006).

Feathers cleaned in our experiment became lighter (brighter). The most probable explanation of this finding

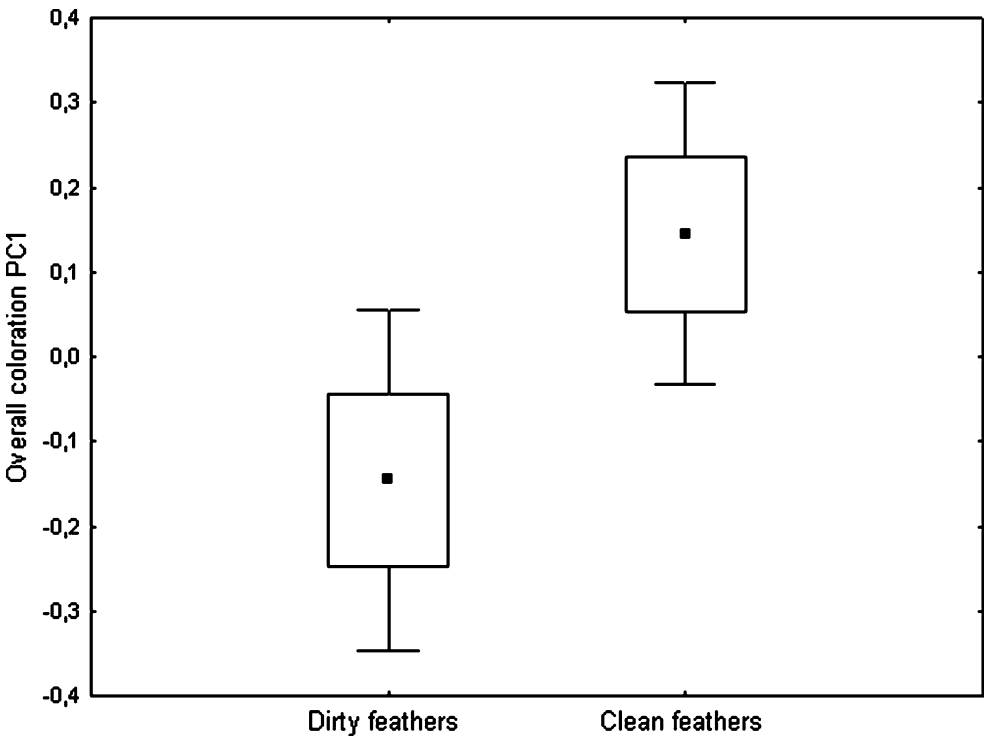

Fig. 1 Overall coloration (*PC1*) of the control (*dirty*) and cleaned feathers sampled from the same individuals. Higher principal component scores indicate more ornamented individuals. *Points*, *bars*, and *whiskers* represent means, SE, and 1.96 SE, respectively

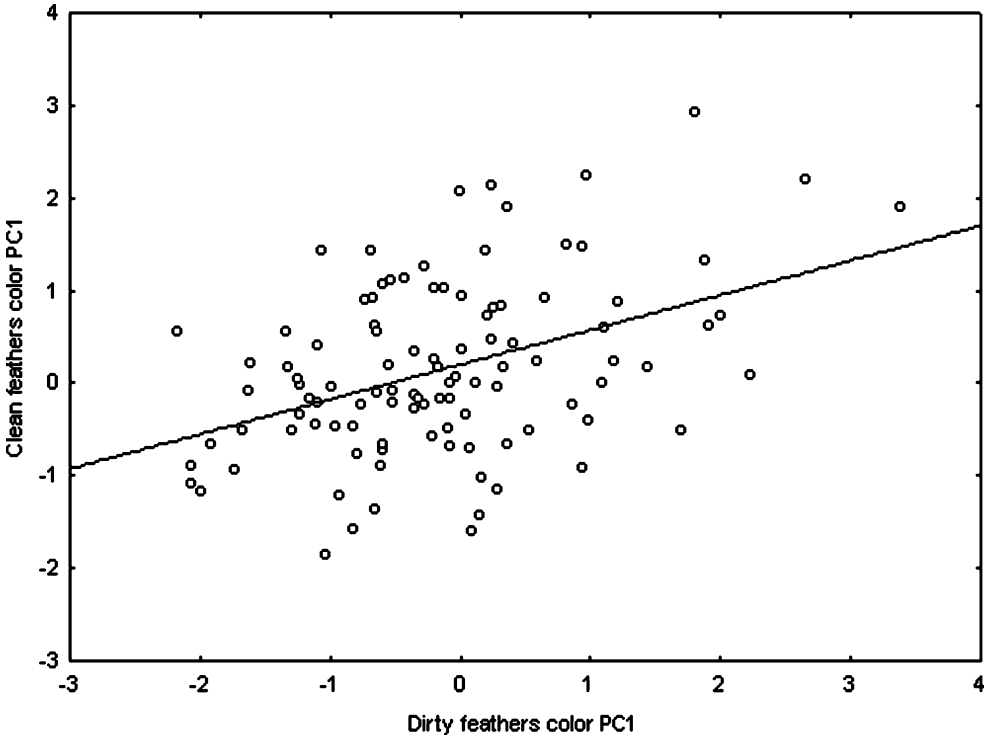

Fig. 2 Relationship between overall coloration (*PC1*) for control (*dirty*) and cleaned feathers. Each *point* represents feathers sampled from the same individual bird

is that all light-absorbing objects attached to the feather surfaces (including preen waxes) decreases the feather's overall reflectance. Experiments by Montgomerie (2006) showed that cleaned feathers exhibited higher total reflectance comparing to dirty ones, irrespective of species and type of coloration. It is important, however, to emphasize that the effect of preen waxes and soiling on achromatic coloration may differ. It has been suggested that preen oils may cause plumage to become glossier and, thus, increase its lightness (Blanco et al. 1999). This hypothesis was supported recently by positive relationship between uropygial gland and plumage brightness reported in great tits (Galván and Sanz 2006).

Our survey demonstrates a possible mechanism, other than feather abrasion, responsible for changes in plumage coloration after moult. In a Spanish population of great tits, researchers observed that the yellow ventral coloration exhibited a reduction in hue, saturation, and brightness values throughout the breeding season (Figuerola and Senar 2005). These changes in hue can be interpreted as the hue shifting toward longer wavelengths later in the season. Our experiment suggests that the changes in hue and brightness indicated by Figuerola and Senar (2005) were likely caused by the accumulation of dirt and waste preen wax during the year. On the other hand, the reduction in saturation observed in the study may have been caused by other factors (e.g., photobleaching; see McGraw and Hill 2004).

Irrespective of what factors caused color change in our study (soiling or preen wax), our results suggest that soil and wax may negatively affect the quality of ornamentation after moult. Color quality of carotenoid-based plumage may have a large influence on the mating prospects of individuals. Females of many species prefer the males that exhibit the more-ornamented coloration, and females may gain some benefits from pairing with the more-colorful males (Griffith and Pryke 2006). Earlier studies provide strong evidence that clean plumage might be an honest signal of male condition. Plumage maintenance constitutes a significant part of birds' daily time budget (Contgreave and Clayton 1994), and only individuals in good condition are able to afford to preen sufficiently (e.g., Yorinks and Atkinson 2000). Consequently, soiled plumage significantly reduces the mating prospects of males (Zampiga et al. 2004). According to our results, soiling/preen wax accumulation influences the carotenoid-based coloration of great tits such that hues become shifted toward longer wavelengths. This effect of wax/soiling accumulation on feathers is similar to the effect of the presence of hemoparasites and

Table 1 Results of repeated-measures ANOVA comparing color (PC1) of the control (dirty) and cleaned feathers taken from the same individual

	F	df	*p* value
Tests of within-subjects effects			
Cleaning	9.00	1.94	0.003
Cleaning × locality	1.72	1.94	0.193
Tests of between-subjects effects			
Intercept	0.57	1.94	0.452
Locality	1.24	1.94	0.269

poor nutritional condition during feather growth on carotenoid-based coloration of great tits (Hörak et al. 2001; Senar et al. 2003). Because the hue of breast plumage is likely to be a target of sexual selection in this species (Hörak et al. 2001), it is reasonable to suggest that feather soiling may reduce male-mating prospects. Our data showed, however, that although impact of soiling and preen wax on coloration was significant, the differences between "clean" and "dirty" feathers were small (hue, 1.4%; brightness, 3.2%). Moreover, the strong relationship between feather coloration pre- and post-cleaning (Fig. 2) indicates a strong individual component to plumage coloration and suggests that preen wax/soiling load may not strongly influence variation among males. Thus, we may assume that, at least in the case of this study, soiling and preen waxes may have minimal effects on sexual signaling. However, it is important to note that the effect of soiling on plumage color may be highly temporal and change with location. Our data were gathered in the beginning of the breeding season, but color change increases as the season progresses (McGraw and Hill 2004; Figuerola and Senar 2005), which may be a consequence of the accumulation of dirt.

Our results indicate that objects attached to feathers contribute to a part of the observed variation in carotenoid coloration. This finding suggests that future work on the subject is warranted. Next studies should devise new methods to enable researchers to distinguish between the effects of preen oil and soiling on plumage coloration. Moreover, to date, there is virtually no information regarding variation in soiling load and soil composition in wild birds. There is a wide array of potential objects that may be attached to feather surfaces (e.g., mites and their droppings, epidermal waste, plant litter, and sand grains), and each may influence feather color in a different way. Research on the absorbance spectra of soiling and preen waxes should be conducted (see Reneerkens and Korsten 2004). The presence plumage soiling and preen waxes has potentially significant implications for mate choice and consequently individual's fitness (see Zampiga et al. 2004). However, future experiments with soiling/preen wax load manipulation within their natural range of variation are needed.

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