

Oxidative stress does not influence carotenoid mobilization and plumage pigmentation

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Oxidative stress has been suggested to create a link between ‘good genes’ and carotenoid coloration via an allocation conflict between external pigmentation and internal antioxidant functions. However, although carotenoid displays have been extensively investigated, there are no experimental tests of the antioxidant efficiency of carotenoids *in vivo*. We induced oxidative stress in a small passerine (the great tit, *Parus major*) under both carotenoid deprivation and supplementation, and investigated the effect on carotenoid mobilization (i.e. plasma) and allocation (i.e. deposition in feather incorporation and liver storage). We found no effects of the stressor on either mobilization or allocation of carotenoids. These results reject the previously suggested superior role of carotenoid’s function as antioxidant *in vivo* with important implications for signal content and honesty.

Keywords: great tit; lutein; *Parus major*; paraquat; reactive oxygen species

1. INTRODUCTION

Over the last decade, immunobiology has been recognized as a fundamental component of evolutionary ecology by providing functional links between allocation trade-offs and costs of, for example, reproductive investment and sexual advertisement (e.g. Sheldon & Verhulst 1996; Norris & Evans 2000; Schmid-Hempel 2003). Carotenoids have played a pivotal role in this framework owing to their dietary origin and multiple suggested roles as immuno-enhancers, pigments and reproductive resources (e.g. Møller *et al.* 2000; Blount 2004; Peters *et al.* 2004). For example, under the (largely untested) assumption that carotenoids are limiting under natural conditions, a positive effect on immunity can form the basis for a trade-off between sexual advertisement through plumage or skin pigmentation and immune function that forms an integral part of honest signalling theory in sexual selection (e.g. Folstad & Karter 1992; Lozano 1994; von Schantz *et al.* 1999). More specifically, in an important and influential paper, von Schantz *et al.* (1999) pointed out that carotenoids could serve as free radical scavengers and, hence, are crucial for combating oxidative stress. One of the attractive features of this hypothesis is its generality, since oxidative stress can derive from external (e.g. parasitic infection or anthropogenic pollution) as well as internal challenges (e.g. metabolic stress) and therefore may be the central currency of many trade-offs in sexual selection and life-history evolution. For example, we have shown that great tits living in an urban environment have a less carotenoid-pigmented plumage and are more oxidatively stressed than rural birds (Isaksson *et al.* 2005), which raised the question whether oxidative stress directly reduce pigmentation as envisioned by von Schantz *et al.* (1999).

The central role of carotenoids in ecological immunology notwithstanding, the hypothesized carotenoid constraints and antioxidant functions remain largely

untested. In fact, recent studies question that carotenoids are important immuno-stimulators or antioxidants (Alonso-Alvarez *et al.* 2004; Costantini *et al.* 2006; Tummeleht *et al.* 2006; Smith *et al.* 2007; Isaksson *et al.* *in press b*). Consequently, they may be too marginally important as antioxidants to mediate significant life-history trade-offs, especially in birds where circulating levels are much higher than in, for example, humans and mammals (Hill 1999; Navara & Hill 2003). Obviously, we need experimental tests to evaluate the effect of oxidative stress on carotenoid levels and the corresponding carotenoid-based phenotypic expression, such as plumage coloration. Here we present the results from a study on a small passerine, the great tit, *Parus major*, using a factorial design to address (i) whether carotenoid intake during feather growth has a positive effect on plumage coloration, (ii) whether circulating levels of plasma carotenoids and liver carotenoid storage are reduced under oxidative stress, and (iii) whether this causes a reduced allocation to plumage pigmentation and, hence, a less chromatic plumage colour.

2. MATERIAL AND METHODS

The great tit, *P. major*, has been extensively used in studies in evolutionary ecology, including links between plumage coloration and life-history investments (e.g. Slagsvold & Lifjeld 1985; Fitzte *et al.* 2003; Tschirren *et al.* 2003; Isaksson *et al.* 2005, 2006, *in press b*). Great tits have a carotenoid-based (mainly lutein and zeaxanthin) yellow ventral plumage (Partali *et al.* 1987; Stradi *et al.* 1998). Previous studies provide baseline data on natural variation in carotenoid content of prey, circulating plasma levels, feather content and spectrometry-based coloration (Isaksson *et al.* 2005, 2006, *in press b*), which allow us to test the role of dietary carotenoids experimentally under oxidative stress while retaining biological realism.

For a controlled exposure to oxidative stress resembling that of urban pollution, we gave captive juvenile great tits paraquat (PQ) in the drinking water, at sub-lethal levels determined by a pilot experiment on birds from the same populations (see §2a). PQ is a common broadleaf weed

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control and is known to induce oxidative stress in plants, humans and other animals (e.g. Autor 1977; Suntutres 2002). The main mechanism of PQ toxicity is through the production of the superoxide anion, which, internally, catalyses the formation of additional reactive oxygen species (ROS), such as hydrogen peroxide and the hydroxyl radical. This induction of oxidative stress puts great emphasis on the use of antioxidants for PQ resistance (Suntutres 2002) and makes it suitable for testing the efficiency of carotenoids in this respect. Furthermore, irrespective of intake route, PQ accumulates in the internal organs (Autor 1977) and creates respiratory oxidative stress as inhaled air pollution (Lorz & Lopez 1997; Dales *et al.* 2006; de Kok *et al.* 2006), which makes the experiment a direct test of the role of pollution-induced oxidative stress for our previously documented variation in plumage colour between urban and rural birds (Isaksson *et al.* 2005).

(a) *Pilot study of paraquat dosage*

A pilot study was conducted on adult great tits ($n=9$) to try out a suitable experimental PQ dosage in the drinking water. Five different concentrations were used (1.5, 0.75, 0.38, 0.19 and 0.09 g l^{-1}) and one female and one male were used in each treatment, except for the highest concentration where only one male was used. PQ is tasteless and scentless, and there was no difference in the amount of water drunk per bird and day between the different concentrations (3.4, 3.4, 3.9, 3.15 and 2.65 ml d^{-1} , respectively). The second lowest (0.19 g l^{-1}) and the lowest (0.09 g l^{-1}) concentrations were not lethal during the six-week-long pilot study. However, birds on the second lowest concentration showed symptoms such as fatigue and lack of alertness. Since we wanted to induce a low chronic oxidative stress, without strong external symptoms, we selected the lowest concentration (0.09 g l^{-1}) for the experiment. This equals an intake each day of approximately 5–10 mg per kilo body weight (the LD_{50} , a single dose of 970 mg per kilo is for Japanese quail, *Coturnix coturnix*; <http://extoxnet.orst.edu/pips/paraquat.htm>).

(b) *Experimental set-up*

Four to five sibling great tits from each of 22 broods ($n=101$) were captured just before fledging (days 18–20 post-hatching). In the aviaries, they were placed together with siblings from two other broods (i.e. total $n=12$) in a nest-box. They were fed by an adult great tit (either male or female) until they were able to feed on their own after approximately two weeks. Birds were held in captivity eight weeks before the onset of the experiment. Two weeks prior to the experiment, the siblings were divided into four experimental groups: (i) carotenoids (Car), (ii) PQ and carotenoids (PQ+Car), (iii) PQ, and (iv) control (Contr). Two cages per treatment were used, with 11 birds in each cage. The two non-supplemented groups (PQ and Contr) were given mealworms fed on potato flakes (carotenoid concentration in these mealworms was $0.03 \mu\text{g g}^{-1}$). The two carotenoid-supplemented groups were given mealworms fed on potato flakes and polenta mixed with lutein and zeaxanthin (carotenoid concentration in these mealworms was $10.51 \mu\text{g g}^{-1}$), which is within the range of foliovorous caterpillar in the wild ($9.4\text{--}69.6 \mu\text{g g}^{-1}$; Isaksson & Andersson 2007). All treatment groups received similar amounts of food (approx. 5 dl mealworms per cage and day, ad libitum), mixed with a carotenoid and vitamin A deficient premix of vitamins, minerals, amino acids and trace elements (iron, copper,

Lactamin, Sweden). The light regime in the aviaries were 16.5 L : 7.5 D before the experiment, and on the experimental day the light regime was changed to 14.5 L : 9.5 D, to simulate natural conditions during moult.

On the first experimental day, morphometry (tarsus length and body mass) and plumage reflectance were measured (see §2c) and a small blood sample ($100 \mu\text{l}$) was taken. All yellow breast feathers from the l.h.s. were plucked to induce regrowth during the experiment. PQ was mixed into the drinking water (0.09 g l^{-1} ; see §2a) of the PQ and PQ+Car experimental groups and was replaced every day. There was no difference in water consumption between birds given PQ water and normal water (C.I. 2007, personal observation). After three weeks (21 days), the experiment was ended, taking new blood samples, feather samples, reflectance and morphometry. The birds were then sacrificed (using decapitation) and the liver was dissected, weighed and frozen at -80°C until high-performance liquid chromatography (HPLC) analysis. The study was conducted in full compliance with Swedish laws and regulation, including ethical permit from Centrala Försökdsnämnden (CFN).

(c) *Plumage reflectance*

Three coincident normal (CN; Andersson & Prager 2006) spectral reflectance measurements (removing the probe between each) were taken from the yellow flank plumage, just below the white bar on the folded right wing, using a USB2000 spectrometer system (Ocean Optics, Inc., Dunedin, USA) and C-spec software (Ancal, Inc., Las Vegas, USA). Before each individual was measured, a dark current and a white reference scan (WS-2, more than 98% uniform reflectance across wavelengths 300–800 nm) were obtained (for further details, see Andersson & Prager 2006). From the raw spectral reflectance data, we computed, and averaged for each individual, the objective colorimetric measure ‘carotenoid chroma’ ($R_{700}-R_{450}/R_{700}$), which, in addition to perceived yellow ‘chroma’, is the best spectroscopic estimate of unsaturated carotenoid pigmentation (Andersson & Prager 2006).

(d) *Carotenoid extraction*

Feather carotenoids were extracted and analysed from the Car ($n=20$) and the PQ+Car ($n=19$) groups, both before and after the experiment. In addition, feathers from five control birds were analysed to assure that the greyish feathers of the carotenoid-deprived groups had no or only small amounts of carotenoids in their plumage (table 1). Carotenoid extraction was performed following Stradi *et al.* (1995) with a few modifications. Briefly, approximately 1 mg of coloured barbs was trimmed off and homogenized in MeOH, with a Retsch MM 2000 micronizer (Hann, Germany) followed by filtering, evaporation under nitrogen, resuspension in the mobile phase (70 : 30 acetonitrile : methanol) and immediate analysis by HPLC (see §2e).

Carotenoids from 188 plasma samples were analysed (39 wild, 90 pre-treatment and 59 post-treatment). The day before the analysis, $20 \mu\text{l}$ plasma was mixed with $380 \mu\text{l}$ acetone and frozen overnight at -80°C . Samples were then (as previously mentioned) filtered, evaporated and resuspended in the mobile phase.

Sixty-five post-treatment liver samples (20 Car, 19 PQ+Car and 19 Contr) were defrosted and homogenized by sonication (Branson ultrasonics corp. Danbury, CT, USA) in EtOH, followed by centrifugation, filtering, evaporation and

Table 1. Mean values of carotenoid chroma, plasma ($\mu\text{g ml}^{-1}$), feather ($\mu\text{g g}^{-1}$) and liver ($\mu\text{g g}^{-1}$) carotenoid concentrations, after the experiment (Car, carotenoid-supplemented; PQ, paraquat in drinking water; contr, control).

	Car			PQ+car			PQ			contr		
	mean	s.e.	N	mean	s.e.	N	mean	s.e.	N	mean	s.e.	N
carotenoid chroma	0.51	0.03	23	0.47	0.03	23	0.15	0.01	19	0.13	0.01	22
feather carotenoid concentration	20.7	2.97	23	17.87	2.46	22				0.41	0.04	5
plasma carotenoid concentration	8.02	1.41	15	6.53	1.00	19	0.11	0.02	10	0.091	0.02	15
liver carotenoid concentration	1.52	0.17	22	1.05	0.22	23				0.02	0.002	19

resuspension as previously mentioned, except that 10 μl THF was mixed with 90 μl of the mobile phase.

(e) HPLC analysis

Samples (20–80 μl) were run isocratically at a 0.5 ml min^{-1} flow through an RP18 column, fitted on a ThermoFinnigan HPLC system (P4000 ternary pump, AS3000 autosampler, UV6000 detector). Chromatograms were analysed with CHROMQUEST v. 4.0 software (ThermoFinnigan, San Jose, USA). Major pigment fractions were identified and quantified by comparisons with standards and calibration curves of lutein (β,ϵ -carotene-3,3'-diol) and zeaxanthin (β,β -carotene-3,3'-diol), provided by Roche Vitamins, Inc., (Basel, Switzerland). Carotenoid concentrations were calculated as the total amount of carotenoids per gram breast feather or liver ($\mu\text{g g}^{-1}$), per liver ($\mu\text{g per liver}$) and per millilitre plasma ($\mu\text{g ml}^{-1}$).

(f) Statistical analyses

Owing to large differences in both means (all $p < 0.0001$) and variances of the key variables (plumage coloration, plasma and liver carotenoid concentrations) between carotenoid-depleted (Contr and PQ) and carotenoid-supplemented (Car and PQ+Car) birds (table 1), the two diet regimes were analysed separately. In cases with two siblings on the same treatment, we used the mean to avoid unbalanced data.

We used restricted maximum-likelihood (REML) linear mixed models, with backward elimination of factors at $p > 0.25$ (Quinn & Keough 2002). Treatment and cage (nested within treatment) were considered fixed. All statistical tests included family as a random factor, and F and p for fixed factors refer to models controlling for family effects. Condition, clutch size, plasma carotenoid concentration or lutein : zeaxanthin (lut : zx) ratio (for feather and liver) and haematocrit (for plasma) were used as covariates in the models. Means are presented with \pm s.e. All statistical analyses were performed in JMP v. 5.1 (SAS Institute, Inc., 2003, North Carolina).

3. RESULTS

(a) Plumage coloration

There was no significant variation in carotenoid chroma among the four treatment groups before the experiment ($F_{3,73} = 1.187$, $p = 0.321$, $n = 76$).

Mean values of carotenoid chroma for the different treatment groups after the experiment are shown in table 1. Carotenoid chroma was not significantly affected by the treatment either among the yellow-coloured

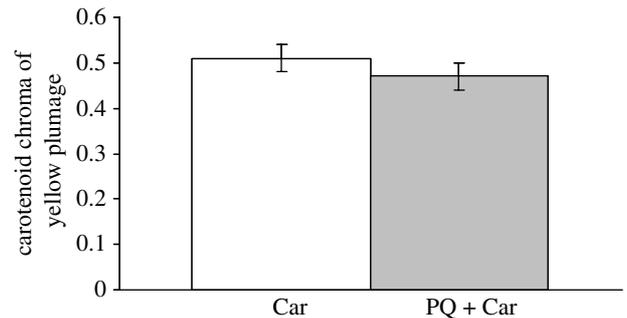


Figure 1. Comparison between carotenoid (Car) and carotenoid plus stressor (PQ+Car) treatments in carotenoid chroma of yellow plumage. Mean \pm s.e.

carotenoid-supplemented groups (Car and PQ+Car: treatment, $F_{1,27} = 1.391$, $p = 0.249$; plasma carotenoids, $F_{1,27} = 14.115$, $p = 0.0008$; figure 1) or among the greyish-coloured non-carotenoid groups (PQ and Contr: treatment, $F_{1,7} = 0.712$, $p = 0.427$; plasma carotenoids, $F_{1,7} = 6.492$, $p = 0.038$).

There was no difference between carotenoid chroma from the feathers developed in the wild and carotenoid chroma after the experiment (mean carotenoid chroma from wild nestling feathers: 0.541 ± 0.008 ; and from carotenoid-supplemented groups after treatments: 0.501 ± 0.119 ; $n = 40$, correlation = 0.024, $p = 0.884$).

(b) Feather carotenoid concentration

Similar to carotenoid chroma, there was no difference in feather carotenoid concentration between the treatment groups before the experiment ($n = 46$, $F_{1,24} = 2.675$, $p = 0.089$). Among all post-treatment carotenoid-supplemented birds (Car and PQ+Car), feather carotenoid concentration and the estimated carotenoid chroma were highly correlated ($n = 40$, correlation = 0.657, $p < 0.0001$).

Mean feather carotenoid concentrations after the treatment are shown in table 1. In line with the results on carotenoid chroma, we found no effect of the stressor on carotenoid incorporation into feathers (Car and PQ+Car treatment: $F_{1,26} = 1.137$, $p = 0.296$; plasma carotenoids post-treatment: $F_{1,26} = 29.658$, $p < 0.0001$). Moreover, there was no composition difference in the feathers between the two most common carotenoids, lutein and zeaxanthin (lut : zx; $n = 28$, Car and PQ+Car treatment: $F_{1,26} = 2.130$, $p = 0.156$; plasma lut : zx ratio, $F_{1,26} = 3.834$, $p = 0.061$).

The mean post-treatment feather carotenoid concentration was $20.02 \pm 2.08 \mu\text{g g}^{-1}$ ($n = 40$), which is half the

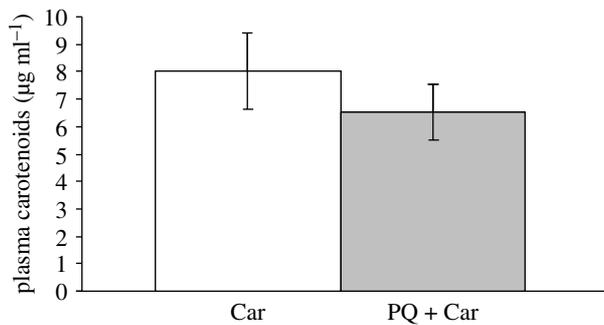


Figure 2. Comparison between carotenoid (Car) and carotenoid plus stressor (PQ + Car) treatments in circulating levels of plasma carotenoids ($\mu\text{g ml}^{-1}$). Mean \pm s.e.

concentration of the original feathers developed in the wild ($40.69 \pm 1.53 \mu\text{g g}^{-1}$, $n=40$). There was no bivariate relationship between the carotenoid concentration from wild and experimental feathers ($n=40$, correlation = 0.174, $p=0.282$).

(c) Plasma carotenoids

There was no difference between the four treatment groups in plasma carotenoid concentration prior to the experiment (treatment: $F_{3,15}=0.736$, $p=0.547$). The plasma concentrations of carotenoids in the carotenoid-supplemented groups ($7.28 \pm 0.89 \mu\text{g ml}^{-1}$) were within the natural range ($0.60\text{--}82.55 \mu\text{g ml}^{-1}$) of wild great tits (Isaksson 2007).

Mean post-treatment plasma carotenoid concentrations are shown in table 1. Again, it was not influenced by treatment either among the carotenoid groups ($n=30$, treatment: $F_{1,11}=0.263$, $p=0.619$; figure 2) or among the non-carotenoid-supplemented groups ($n=24$, $F_{1,8}=0.896$, $p=0.372$). Cage and body condition were non-significant and excluded from both models. The lut:zx ratio in the plasma was not significantly affected by the treatment ($n=30$, Car and PQ + Car treatment: $F_{1,11}=1.516$, $p=0.244$; $n=24$, PQ and Contr treatment: $F_{1,8}=0.426$, $p=0.532$).

Moreover, post-treatment plasma concentration was significantly positively related to plasma before the experiment (when the birds had been in captivity for eight weeks; $n=29$, correlation = 0.690, $p<0.00001$). The same relationship was shown for the non-carotenoid-supplemented groups (before and after the experiment: $n=24$, correlation = 0.575, $p=0.003$).

(d) Liver carotenoids

Mean liver carotenoid concentrations are shown in table 1. Liver carotenoid content ($\mu\text{g per liver}$) was not influenced by the treatment ($n=27$, Car and PQ + Car: $F_{1,24}=2.087$, $p=0.162$; condition: $F_{1,24}=8.262$, $p=0.008$; plasma carotenoids: $F_{1,24}=59.972$, $p<0.0001$), nor did the treatment affect the carotenoid composition (lut:zx ratio; $n=28$, $F_{1,25}=2.076$, $p=0.162$; lut:zx ratio in plasma: $F_{1,25}=15.492$, $p=0.0006$).

4. DISCUSSION

To our knowledge, the present study is the first controlled induction of oxidative stress *in vivo* to test the hypothesized trade-off between antioxidant defence and plumage pigmentation. Contrary to this hypothesis, however, we detected no effects of the oxidative stressor

(PQ) on mobilization (i.e. circulating levels) or allocation (i.e. pigmentation and storage). This argues against a generally important role of carotenoids in combating oxidative stress and as mediators of honest signalling.

There is substantial evidence that carotenoids are efficient antioxidants *in vitro* (e.g. Mortensen & Skibsted 1997), but the effect *in vivo* is more ambiguous (e.g. Halliwell & Gutteridge 2002; Hartley & Kennedy 2004; Kiokias & Gordon 2004). However, the positive effects of increased carotenoid intake have been documented on, for example, disease resistance, reproductive effort and hatching success (e.g. Surai 2002; McGraw & Ardia 2003; Bertrand *et al.* 2006). However, it is uncertain whether this is via an improved antioxidant function or via other mechanisms (see Hartley & Kennedy 2004). A few recent studies have investigated the relationships between plasma carotenoids and measures of antioxidant protection, capacity or oxidative damage (Alonso-Alvarez *et al.* 2004; Costantini *et al.* 2006; Tummeleht *et al.* 2006; Isaksson *et al.* in press b). None of these studies found plasma carotenoids to correlate with other measures, suggesting that carotenoids may not be as important antioxidants as previously suggested (e.g. von Schantz *et al.* 1999).

In this study, experimental elevation of internal ROS levels via PQ should activate a wide repertoire of antioxidant systems (Suntres 2002; Dotan *et al.* 2004). Thus, if carotenoids are depleted by the defence against oxidative damage, PQ birds should have less circulating carotenoid levels than birds that were not exposed to the stressor, in particular with respect to zeaxanthin, which is proposed to be a better antioxidant than lutein (e.g. Mortensen & Skibsted 1997). Alternatively, carotenoids may be mobilized from tissues, such as the liver, to increase protection in other parts of the body, which could increase plasma concentrations (Costantini & Dell'Omo 2006). However, neither of these scenarios was supported.

Importantly, there was no effect of PQ-induced stress on the regrown yellow plumage pigmentation (based on both spectrometry and feather content analyses). However, there was a strong effect of carotenoid supplementation on both plasma and plumage pigmentation. These were also strongly correlated, suggesting that any depletion or reallocation of plasma carotenoids, in response to the PQ treatment, should have resulted in paler yellow plumage. In other words, while a dependence of plumage pigmentation on plasma carotenoid availability is supported (see also Hill *et al.* 1994), no effect of oxidative stress on either availability or allocation to plumage could be detected. These results are supported by previous work of Navara & Hill (2003), who found no allocation conflict between plumage coloration and immune defence of the two carotenoids of interest here (lutein and zeaxanthin). However, even though there is no direct evidence for a signalling function of this specific yellow plumage (Isaksson *et al.* 2006, in press a), our study questions the generality of the role of oxidative stress as an honesty-maintaining cost of carotenoid-based colour signals in birds.

Furthermore, plasma (but not feather) concentration showed a significant positive relationship between pre- and post-treatment. Because this cannot be explained by differences in carotenoid availability or intake, it suggests that there is either genetic variation in, or early

'programming' effects on, carotenoid assimilation (Barker 1998; Blount *et al.* 2003). However, under natural variation in carotenoid intake, this effect is likely to be diluted by post-hatching environmental effects as suggested by previous field studies (Fitze *et al.* 2003; Isaksson *et al.* 2006).

Although laboratory studies necessarily have to be treated with caution when extrapolating to natural populations, the carotenoid supplementation (via carotenoid-enriched mealworms) was in this study uniquely similar (in both composition and concentration) to the natural diet of caterpillars during breeding (Partali *et al.* 1987; Isaksson & Andersson 2007). This also resulted in plasma and feather concentrations within the natural range, suggesting that our results accurately reflect what would have happened under severe, but non-lethal, oxidative stress in natural populations.

In conclusion, our study does not support the hypothesis that carotenoid pigmentation is traded off against antioxidant defence. Instead, it suggests a rather minor, and not limiting, role of carotenoids as antioxidants *in vivo* in birds (see also Hill 2002; Isaksson *et al.* in press *b*). In this respect, carotenoids may indeed be a red, or in this case yellow, 'herring' (Hartley & Kennedy 2004).

The study was conducted in full compliance with Swedish laws and regulation, including ethical permit from Centrala Försökjurynämnden (CFN).

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