

Fruit for health: the effect of flavonoids on humoral immune response and food selection in a frugivorous bird

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Summary

1. The intake of antioxidants confers health benefits to consumers by reducing oxidative stress and improving immune functions. Therefore, several life-history traits such as growth, immunity, senescence and the expression of sexually selected traits might be affected by dietary antioxidants.
2. Flavonoids are strong antioxidants *in vitro*, and are among the commonest found in fruits, which are a primary source of antioxidants for many animals. It is therefore likely that flavonoids play a beneficial role as dietary antioxidants, but their potential has been ignored in evolutionary ecology.
3. We investigated the ecological importance of flavonoids, using wild-caught blackcaps (*Sylvia atricapilla*), a frugivorous European songbird.
4. We verified the assumption that flavonoids can be absorbed and circulated by blackcaps.
5. In a food selection experiment, we showed that, when given a choice between food with and without fruit flavonoids, blackcaps actively select food with flavonoids.
6. We found a positive effect of flavonoids on humoral immune response. Birds supplemented for 4 weeks with a modest quantity of flavonoids were more likely to mount a humoral immune response after an immune-challenge, compared with control birds.
7. To conclude, our study demonstrates that birds select food with flavonoids and that these antioxidants increase consumer humoral immune response. Since these effects occurred at a moderate dose that is obtainable also by many omnivorous consumers, we suggest that flavonoids may play a beneficial role in ecological immunology for several wild species.

Key-words: dietary antioxidants, ecological immunology, food selection, immunocompetence, plant–animal interaction

Introduction

A major research avenue of the last decade has been to determine how the immune system benefits from the intake of dietary antioxidants. Dietary antioxidants, such as carotenoids and vitamin E are known to increase the immune response in birds or other taxa of vertebrates (Boa-Amponsem *et al.* 2000; Blount *et al.* 2003). The mechanisms underlying this phenomenon are not completely understood, but it is generally hypothesized that the ingestion of antioxidants may reduce the deleterious effects of free radicals on the immune response (von Schantz *et al.* 1999). Given the generality of this mechanism, one might expect that other dietary antioxidants would enhance

the immune system, and that strong and/or common antioxidants have a greater relevance for consumers than weak and/or rare antioxidants.

The best sources of antioxidants for humans and animals are fruits and vegetables, where flavonoids reach extremely high concentrations (Havsteen 2002). Flavonoids are not only among the most common antioxidants in nature, but also those with the highest antioxidant potency *in vitro* (compare Miller *et al.* 1996; Rice-Evans, Miller & Paganga 1996; Gliszczynska-Swiglo 2006). Although their best-described property is to act as antioxidants (Nijveldt *et al.* 2001), flavonoids can display a huge array of biochemical and pharmacological effects that affect the function of the immune system and inflammatory processes (Middleton & Kandaswami 1992). Due to their antioxidant, antiviral, anti-allergic and anti-inflammatory activities, they are considered ‘health-promoting,

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disease-preventing dietary compounds' (Middleton 1996). It is therefore not surprising that numerous medical studies have focused on the health-enhancing effects of flavonoid consumption, with more than 30 000 publications per year in the last few years (Scalbert, Johnson & Saltmarsh 2005). However, most of these studies are *in vitro* and, because of the rapid metabolism of flavonoids after ingestion, their results may not completely apply to living systems. Therefore several authors pointed out the need for studies using *in vivo* supplementations of purified flavonoids (e.g. Scalbert *et al.* 2005; Williamson & Manach 2005).

Many species of free-ranging birds (Fleming, Breitwisch & Whitesides 1987; Corlett 1998) ingest large amounts of fruits and are therefore optimal candidates to study the health benefits of flavonoid ingestion *in vivo*. Recently, Schaefer, McGraw & Catoni (2007) demonstrated that the blackcap (*Sylvia atricapilla*), a frugivorous bird, actively selected food enriched with anthocyanins, a class of flavonoids, when offered a choice against an otherwise identical food, but which lacked anthocyanins. However, the benefits that wild consumers may reap by selecting anthocyanins, or flavonoids in general, are still hypothetical. This is because flavonoids have not been incorporated into ecological immunology yet, which aims to determine the costs of and the variation in immune defences of wild animals. This is surprising because the immune-enhancing effects of dietary antioxidants (mainly carotenoids, but also melatonin, vitamins C and E) have been a major focus in both behavioural ecology and ecological immunology in the last decade (Blount *et al.* 2003; McGraw & Ardia 2004; Bertrand, Faivre & Sorci 2006).

Here we evaluate the benefits that wild consumers may gather from the ingestion of flavonoids, using the blackcap as a model species, as in Schaefer *et al.* (2007). First, we tested the basic assumption that anthocyanins and quercetin, the two most common flavonoids in our extracts, can be absorbed and circulated by birds. Then, we tested whether birds prefer food enriched with a mixture of fruit flavonoids. Finally, and most importantly, we tested the hypothesis that birds can obtain immunological benefits from the ingestion of flavonoids.

Methods

The blackcap is a European migratory species, and frugivory plays an important role in its ecology and life-history (Eggers 2000). Birds were captured in mist-nets at the Vogelwarte Radolfzell (Germany, 47°46'07" N, 8°59'44" E), and sexed and aged (first year, second year or older) following Shirihai, Gargallo & Helbig (2001).

ABSORPTION STUDY

In a pilot experiment between 12 and 27 October 2004, we established whether birds can circulate flavonoids in blood, a prerequisite for any activity of dietary antioxidants at the organismal level. Birds (10 males and 7 females) were housed in small cloth cages (20 × 20 × 21.5 cm) and fed *ad libitum* with mealworms (*Tenebrio molitor*) and locally collected elderberries (*Sambucus nigra*). These fruits are consumed by free-ranging blackcaps (Eggers 2000) and contain a high concentration of flavonoids (Määttä-Riihinen *et al.* 2004).

Using heparinized capillaries we collected a blood sample ($\pm 75 \mu\text{L}$) from the wing vein of each bird at capture and one blood sample a week later upon release. Blood was centrifuged immediately, plasma separated from the cells, and stored at -70°C . Owing to the small amount of plasma available, we pooled all samples.

By following exactly the protocol of Cao & Prior (1999), we extracted anthocyanins from plasma using an ODS solid-phase extraction cartridge (Sep-Pak C18). The cartridge was washed with 10 mL of methanol and equilibrated with 10 mL of 0.44 mol L^{-1} TFA before use. The available plasma was diluted with 200 mL of 0.44 mol L^{-1} TFA and applied to the cartridge. Water-soluble compounds, polar lipids and neutral lipids were eluted from plasma samples with 10 mL of 0.44 mol L^{-1} TFA, 10 mL of dichloromethane and 10 mL of benzene, respectively. Samples were finally eluted with 5 mL of 0.44 mol L^{-1} TFA in methanol to collect anthocyanins. The methanol phase was collected, evaporated to dryness with a rotary evaporator and redissolved in 200 mL of 0.44 mol L^{-1} TFA aqueous solution. Finally, 80 mL of the TFA aqueous solution, which contained the redissolved anthocyanins, was injected into an HPLC system to determine anthocyanin concentrations. For HPLC and MC analysis we followed exactly the protocol of Schwarz *et al.* (2003). We also quantified the plasma concentration of anthocyanins in a HPLC system using cyanidin 3-glucoside as reference following Cao & Prior (1999).

BIRD MAINTENANCE

The experiments were part of a larger project on the effects of dietary flavonoids on life-history components of blackcaps that was performed during two subsequent seasons (April–October, when blackcaps are present at our latitude). In 2005, birds were captured between 7 and 27 April and, after a habituation period of 10–20 days with live mealworms, were maintained on a diet consisting mainly of crushed rusk, egg-whites and soured milk (8.1% carbohydrates, 4.0% proteins and 2.7% lipids per wet mass – for the exact composition see Appendix S1 in Supplementary material). In 2006, birds were captured between 27 March and 27 April, and from 8 May, were fed with a semi-synthetic food optimized for small omnivorous birds, containing casein, glucose, amino acids, cellulose, agar and plant oil (for exact composition, see Pierce *et al.* 2005). None of the two food types contained berries, berry-derivatives or sheep-based products. We decided to switch to a different diet in 2006 in order to test the effect of flavonoids on the immune system under different dietary conditions, and to enhance control over the intake of other antioxidants.

Birds (sex ratio 1 : 1 in both years) were held in wire cages (40 × 35 × 30 cm) covered with a thin white cotton cloth in 2005 and in larger cages (40 × 70 × 30 cm) with cloth sides and ceiling in 2006. Birds were kept under natural photoperiod and constant temperature of 20 °C. In the course of all experiments described here, no mortality occurred and birds maintained good body condition (body mass before 16.8 ± 0.1 (mean \pm SE), after 16.5 ± 0.1 ; haematocrit before 46.7 ± 0.4 , after 45.6 ± 0.5). All values were well within the limits found in literature (see e.g. Cramp, Simmons & Perrins 1977–1994; Campbell 1994). In 2006, however, 23 birds died between late July and December. These animals suffered mostly from wing and bill injuries. The exact cause of the death of the animals remains unknown.

FLAVONOID EXTRACT

Flavonoids are mostly water-soluble polyphenols (Eastwood 1999). By following exactly the protocol of Schwarz *et al.* (2003), flavonoids

were extracted with an extraction column filled with Amberlite XAD-7 as solid phase (Fluka, Buchs, Switzerland), and methanol and distilled water as mobile phases. We did not remove non-coloured polyphenols, such as flavanols, in order to obtain an extract with a composition in flavonoids as similar as possible to that of berries. Methanol traces were completely removed from our extracts by evaporation *in vacuo*. Because this methodology has been developed by Schwarz *et al.* (2003) to produce flavonoid extracts for human consumption, we believe that the presence of methanol in the extracts was not a concern in our study.

In 2005, we extracted flavonoids from blackberries (*Rubus* sp.), while in 2006 we used bilberries (*Vaccinium myrtillus*). Extracts consisted of 50 g/100 g (2005) and 35 g/100 g (2006) of anthocyanins, while quercetin, myricetin and other minor flavonols accounted for 16 g/100 g of the extracts and condensed tannins made up the rest, thus mirroring the natural flavonoid content of wild berries. By using extracts obtained from two different species we could test the generality of the beneficial effects of flavonoids on consumer antibody production. In both years, the extracts were stored in daily servings in Eppendorf tubes at -20°C and were mixed in the food every morning a few minutes before the food was offered to the birds.

DIETARY TREATMENT

In both years, we assigned birds randomly to two diets starting 8 May and ending upon release. Each bird of the flavonoid group received a daily average of about 2.8 mg flavonoids (corresponding to about 260 mg kg⁻¹ of body mass) mixed in the food, whereas the control group ate the same food without flavonoid extract. The dietary treatment prior to the SRBC experiments lasted 31 and 44 days in 2005 and 2006, respectively. The concentration of flavonoids in the food was 622 mg kg⁻¹ in 2005 and 304 mg kg⁻¹ in 2006. The higher water content of the semi-synthetic food used in 2006 was responsible for differences in flavonoid concentration and food consumption between years (see below). The amount of flavonoids supplemented was chosen in order to reflect our estimates of flavonoid ingestion in blackcaps during periods of relatively low frugivory (< 10 berries a day).

Experimental and control groups consumed a similar amount of food. Average daily food consumption in 2005 ($n = 4$ samples per bird ($n = 60$), taken 21 May, 17 June, 17 July and 15 August): control group: 4.6 ± 0.17 g (mean \pm SE), flavonoid group: 4.5 ± 0.17 g; t -test: $t = 0.69$, $P = 0.5$. Average daily food consumption in 2006: control group ($n = 5$ samples per bird ($n = 60$), 7, 15 and 24 June, 4 and 18 July): 9.2 ± 0.12 g, flavonoid group: 9.1 ± 0.12 g; t -test: $t = 0.33$, $P = 0.7$.

FOOD CHOICE EXPERIMENT

To establish whether blackcaps consistently select food containing flavonoids, we conducted a first food-choice trial during the breeding season (18–21 June 2005) and a second one during moult (28–31 August 2005). For the first trial, we used 60 birds, whereas for the second trial we used 47 birds. The remaining 13 birds were treated (end of August 2005) with an anti-coccidian drug and were left out of the experiment. During the food choice trial, starting at dawn, we provided two paper cups (4×2.5 cm) each containing 4.4 ± 0.05 g (mean \pm SE) of standard food, one with 2.8 mg of flavonoids added. The two foods were of identical consistency, but the food with flavonoids was darker (see Supplementary Appendix S2). After 7 h we removed and weighed the cups and calculated food intake, adding the occasional spills to the respective cups and correcting for evaporation using two control food cups. Four and one birds

were excluded from the analysis of the first and second trial, respectively, because they spilled all food on 2 days.

We compared the intake of the two food types with a repeated measures model. To test the consistency of bird food choice, we included in the analysis only those birds that had complete data for both trials ($n = 44$). First, we obtained a daily 'Δ food intake' for each bird, by calculating the difference between the amounts of ingested food with flavonoids minus the ingested control food. This new set of eight variables (Δ food intakes of each bird for 8 days) was then used as dependent variable in the repeated measures model. Dietary pre-treatment (i.e. whether birds were or not supplemented with flavonoids before the start of the food-choice experiment), sex, age, trial (June or August) and their interactions were factors. To check if dietary conservatism affected the results, we tested whether birds of both experimental groups (flavonoid supplemented and control) selected food with flavonoids with two t -tests.

IMMUNE EXPERIMENT

We tested the effect of the supplementation with flavonoids on the immune system in 40 birds in 2005 and 60 birds in 2006 (sex ratio 1 : 1 in both years). Twenty birds in 2005 were used as control and injected with phosphate buffered saline (PBS). We challenged birds with sheep red blood cells (SRBCs, Fiebig Nährstofftechnik, Germany), a non-pathogenic cellular antigen that elicits a humoral immune response, commonly used as an indicator of immunocompetence in birds (Martin, Weil & Nelson 2006). On day 0 (8 and 9 June 2005, 21 June 2006), we collected 40–60 μL of plasma as described above and injected birds intra-peritoneally with 75 μL of fresh 10% SRBCs in PBS. Eight days later we collected another plasma sample. Antibody titre (log concentration) in plasma was determined with a haemagglutination titration assay (Hudson & Hay 1976). Titres were scored as the highest well number with agglutination, subtracting the titres of heterologous antibodies found in the pre-immunization samples ($n = 8$ birds, average = 1.8, similar to Snoeijs *et al.* 2007). One bird in 2005 was excluded from the analysis because we failed to take a post-immunization blood sample. Six birds in 2006 were excluded from the analysis because their samples were lost due to technical problems (freezer failure). More than half of the birds did not produce a detectable antibody response (non-responders), which is common during primary immunizations with SRBCs (e.g. 0–79% of non-responders in non-breeding and breeding zebra finches (*Taeniopygia guttata*, Deerenberg *et al.* 1997), 30–85% in free living and captive superb fairy wrens (*Malurus cyaneus*, Peters 2000) and 100% non-responders in European greenfinches (*Carduelis chloris*, Aguilera & Amat 2007). Therefore, we initially classified birds as responders or non-responders and analysed immune responsiveness as binary (yes/no) in nominal logistic models, with flavonoid treatment, experimental year, age (1-year-old/older) and sex as fixed effects. Interactions were also considered in the model. We then analysed antibody production of responders quantitatively comparing supplemented and control birds in Wilcoxon rank sum tests.

Statistic analyses were performed with JMP IN 5.1 (SAS) and with SPSS 15.0.

Results

ABSORPTION EXPERIMENT

Mass spectrometry confirmed that blackcaps absorb and circulate anthocyanins and quercetin in plasma. We found a plasma concentration of anthocyanins of 24 nmol L⁻¹.

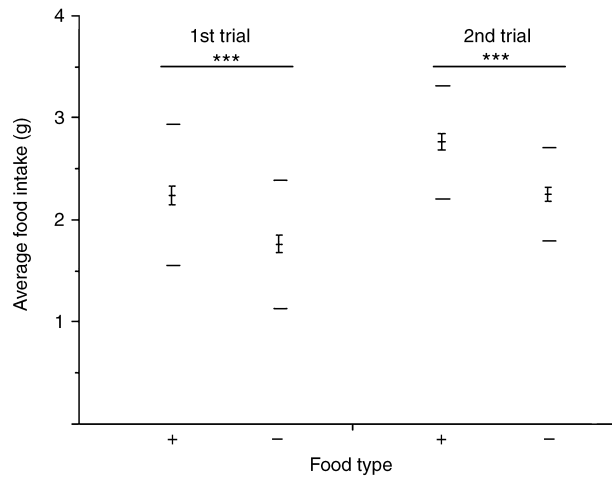


Fig. 1. When given a choice of the same food with (+) and without (-) added flavonoids, blackcaps consumed on average (in grams) more food with flavonoids. Illustrated are mean food consumption (in grams) \pm SE (bounded lines) and SD. *** = $P < 0.001$.

FOOD CHOICE EXPERIMENT

Birds clearly selected food with flavonoids (intercept $F = 33.3$, $n = 44$, $P < 0.001$), ingesting 25% more food with flavonoids, than food without flavonoids (Fig. 1). Mean flavonoid consumption after 7 h was 1.6 ± 0.06 mg. There was no effect of sex ($P = 0.53$) and age ($P = 0.35$) on food selection. The factor trial was not significant ($P = 0.76$) showing that food selection was consistent within birds and between trials. Dietary pre-treatment significantly affected bird food choice ($P = 0.01$). Birds fed with flavonoids ate more food with flavonoids than birds in the control group, suggesting a tendency toward dietary conservatism. Both groups however ate more food with flavonoids than control food (flavonoid fed birds: t -test, $t = 6.06$, $n = 30$, $P < 0.0001$; control birds: t -test, $t = 2.18$, $n = 29$, $P = 0.04$). All interactions between age, sex, trial and pre-dietary treatment were not significant (all $P > 0.15$).

IMMUNE EXPERIMENT

Flavonoid supplementation affected immune responsiveness: 54% ($n = 25$) of supplemented birds produced antibodies, vs. 30% ($n = 14$) of control birds ($\chi^2 = 5.62$; $n = 93$; $P = 0.018$). The proportion of birds showing agglutination in pre-inoculation blood samples was not different between experimental groups (Wilcoxon test; $Z = 0.013$, $P = 0.98$). None of the PBS injected birds in 2005 showed an antibody response. There was no effect of experimental year ($P = 0.57$), sex ($P = 0.62$), age ($P = 0.12$) or their interactions (all $P > 0.15$) on immune responsiveness. Antibody titres produced by responders were not affected by supplementation (average for controls: 1.9; for supplemented birds: 2.2, $Z = 0.1$, $P = 0.9$; titres were within the normal range, cf. Deerenberg *et al.* 1997; Peters 2000; Snoeijs *et al.* 2007).

Discussion

Our study demonstrated for the first time that flavonoids are beneficial compounds that may enhance consumer humoral immune responsiveness *in vivo*. We further demonstrated that wild birds actively select food containing flavonoids. Finally, we found that flavonoids circulate in bird blood at a concentration comparable to that found in humans or rats (range of concentration of anthocyanins in plasma: from 10 to 1280 nmol L⁻¹) (Matsumoto *et al.* 2006; Talavera *et al.* 2006). Our results have important implications for avian ecological immunology and for the evolution of plant–seed disperser interactions.

Daily flavonoid supplementation increased the ability of birds to mount a primary immune response to a novel antigen. The relevant parameter was whether or not to mount an antibody response rather than actual antibody titre, as commonly observed in this immune test (Deerenberg *et al.* 1997; Peters 2000; Hanssen *et al.* 2004). Because we used a different food type and flavonoid extract each year, a limitation of our study is that ‘year effects’ might have masked or amplified any treatment effects. However, the stimulatory effect of flavonoid supplementation was robust occurring in both years, although the ingested flavonoids originated from two different plant species and were added to two different food types with different antioxidant contents.

Positive effects of flavonoids on immune responsiveness might have a variety of underlying mechanisms. Activated immune cells generate free-radicals and increase oxidative stress (Costantini & Dell’Omo 2006; Hřrak *et al.* 2007), while T- and B-cell-based immune reactions are highly sensitive to oxidative damage (von Schantz *et al.* 1999). Flavonoids can reduce oxidative stress by directly scavenging free-radicals, by interfering with free-radical producing mechanisms and by increasing the function of endogenous antioxidants (Nijveldt *et al.* 2001). Additionally, flavonoids may regenerate other antioxidants with known immune-enhancing activity, such as vitamin E (Zhu, Huang & Chen 2000) and carotenoids (Pietta & Simonetti 1998). Finally, *in vitro*, flavonoids can have profound direct effects on a variety of immune and inflammatory cell functions, particularly once cells are activated (Middleton & Kandaswami 1992) with inhibitory as well as stimulatory actions (Middleton, Kandaswami & Theoharides 2000).

Our supplementation corresponds to the daily consumption of 1–2 blackberries, bilberries or elderberries. Our experiment simulated such a modest intake of flavonoids because high quantities of flavonoids are only available during the limited time period of maximal berry abundance (e.g. from August to October in northern temperate climate). During the rest of the year, frugivores have to compete for the fewer fruit resources available (Snow & Snow 1988), and many fruits contain less flavonoids than the species we used for extraction (Moyer *et al.* 2002; Määttä-Riihinen *et al.* 2004). In particular, when we ran our experiments in June, ripe fruits are scarce (e.g. wild cherry *Prunus avium* and honeysuckles *Lonicera* spp.). The importance of flavonoids as immune-stimulants is

thus not limited to animals consuming fruit in large quantities, but might also apply at times when fruits are not plentiful and to omnivores that consume fruits only in modest quantities.

These results not only add to the dietary antioxidants (carotenoids, vitamins) that are known to enhance immune responses in birds (Leshchinsky & Klasing 2001; Blount *et al.* 2003; Faivre *et al.* 2003; McGraw & Ardia 2003; Saino *et al.* 2003), but also places their ecological role in perspective. We hypothesize that the bioavailability (high concentration and high potency) of flavonoids might under certain circumstances obscure the immune-enhancing effect of other dietary antioxidants. Birds might ingest about 2–3 mg of flavonoids with a single berry, whereas other dietary antioxidants such as carotenoids not only have lower antioxidant potency but their daily intake is often < 0.1 mg in small passerines (McGraw 2005). Likewise, presence of flavonoids in captive diets might affect the outcome of supplementation experiments (compare similar caution expressed by Middleton & Kandaswami (1992) on flavonoid content of animal fodder). We suggest that the relative ecological significance of various dietary antioxidants should be reconsidered taking into account the availability and effects of flavonoids, at least for those species that consume, even occasionally, fruits.

Schaefer *et al.* (2007) recently demonstrated that birds are able to visually discern anthocyanin concentration in fruits and that blackcaps actively select food rich in anthocyanins, when given a choice against an otherwise identical food. Here we confirm that blackcaps select food that contains several different types of flavonoids, and thus mirrors flavonoid composition of wild fruits. Furthermore we demonstrate here that birds may reap immunological benefits by selecting food enriched with fruit flavonoids. Although birds showed dietary conservatism, a well-known phenomenon in wild and captive birds (Marples & Kelly 1999), the selection for food with flavonoids was apparent in both experimental groups. In our experiments, birds were able to visually distinguish the food with flavonoids from the control food (see Supplementary Appendix S2). The colour difference was caused by the high proportion of anthocyanins, which are the natural pigments of many fruits, in the supplement. This situation mirrors natural conditions, because the flavonoid content of a fruit is predictable by its colour (Schaefer *et al.* 2007). Since adult and yearling wild-caught blackcaps do not show a colour-preference if food is nutritionally identical (Schmidt & Schaefer 2004; Gamberale-Stille, Hall & Tullberg 2007) we are confident that our results are due to a (learned) selection for flavonoid content and not due to innate selection for darker food *per se*. Because anthocyanin content correlates with the total antioxidant capacity of dark berries (Moyer *et al.* 2002), birds are potentially able to select fruits using their colour as a reliable signal of health benefits (Schaefer *et al.* 2007). We hypothesize that consumers can thus attend to plant signals to increase their intake of flavonoids for self-medication. If birds consume fruits to increase health benefits, plants in turn can increase their reproductive success by advertising the antioxidant properties of their fruits.

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Supplementary material

The following supplemental material is available for this article:

Appendix S1. Food composition for the year 2005

Appendix S2. Spectrometric measurement of food reflectance.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2435.2008.01400.x>

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