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Characterisation of the major carotenoids in the plasma of the Eurasian magpie (*Pica pica*) nestlings

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Since carotenoids can serve as antioxidants and/or immune stimulants, it has been assumed that deposition of carotenoids in feathers or bills can reveal the health status in birds. In order to study the function of carotenoids as signals or immune stimulants, it is necessary to characterise the types of carotenoid molecules in the study species. In this preliminary study, we examined the types of carotenoids in the plasma of four nestlings of the Eurasian magpie (*Pica pica*) using mass spectrometry. We found that lutein or zeaxanthin is the major carotenoid in the plasma of magpie nestlings. Some minor constituents of carotenoids could be regarded as the metabolized forms of lutein or zeaxanthin. The plasma of magpie nestlings contained both yellow carotenoids such as lutein and zeaxanthin and red carotenoids such as astaxanthin, which may result in the varying degrees of redness in the plasma among the individuals.

key words: carotenoid, plasma, nestling, Eurasian magpie, *Pica pica*

Introduction

Carotenoids are one of the major pigments in animals and plants. Because carotenoids cannot be synthesized in animals and must be obtained from the diet (Olson and Owens 1998), they are suggested to function as antioxidants and/or immuno-stimulants (Lozano 1994; Olson and Owens 1998; McGraw 2006; Svensson and Wong 2011; Simons et al. 2012). This implies that the level of carotenoids in animal's body can reflect the health status, such as feeding condition and/or feeding ability, of the individual. Many studies tested this correlation and found that carotenoid contents reveal the health status and/or the quality of the individual (reviewed in Hill 2006, 2011). However, these studies were mostly conducted in birds where carotenoids are used as social signals. Carotenoids can be deposited in the plumage, bill and skin where the coloration is visible to other individuals and thus serves as social signals. Since carotenoids are used as signals in these species, the amount of carotenoids that are available as antioxidants or immuno-stimulants are limited (but see Simons et al. 2014). On the contrary, no study examined the carotenoid content in

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the plumage or in the plasma in those species whose signalling system is not based on carotenoids; since the availability of carotenoids in these species is not limited by the trade-off between signalling and immune function, it is likely that the carotenoids accumulated or circulating in the body of these species is fully involved in enhancing immune system.

In this study, we investigated the types of carotenoids in the plasma of magpie nestlings. Magpies do not contain any carotenoid-based signals and their signalling system heavily relies on melanin (Lee S.-I. unpublished data; Lee et al. 2010). If so, one can predict that, in magpies, there is no trade-off in carotenoids usage between signalling and immune function, and all the carotenoids that magpies have in the body can be used as antioxidant or immuno-stimulant. Before one examines the availability of carotenoid and the immune capacity of individual bird, it is important to know what types of carotenoid molecules are present in the body and how variable the contents are among the individuals. This was the main purpose of this preliminary study. We chose to examine the carotenoids that are present in the blood in the nestlings (i.e. circulating carotenoids). This is because the carotenoids in the body of the nestlings are obtained from the food that the parents provided, which is predominantly based on insects (Birkhead 1991), so we would know the effect of feeding condition of the nestlings. Also, as the nestlings are in the stage of fast growth, examining the types and the level of circulating carotenoids in the blood would provide us some insights on what carotenoids are being delivered in the body (Deviche et al. 2008). The results of this study would serve the basis for any future studies on the total carotenoid content and the function of carotenoid in the birds where carotenoids are not used in social signalling.

Materials and methods

We used four plasma samples that were taken from the nestlings of the Eurasian magpie (*Pica pica*). Information of the nestlings that were used in this study is given in Table 1. The blood samples were taken from the brachial vein and microcentrifuged 3−5 hrs after collection for 3 mins to separate blood cells and plasma.

Table 1. Information of the nestlings that were sampled in this study.

ID	Sampled year	Nest ID	Age at sampling (days after hatching)	Sex	Weight (g) at sampling	Tarsus length (mm) at sampling
301B	2013	301	25	M	200.4	52.71
40W	2013	40	25	F	186.0	51.98
117Bk	2011	117	7	F	24.8	14.77
117Y	2011	117	7	F	26.6	15.40

We conducted mass spectral analyses on these four plasma samples together with lutein standard solution and astaxanthin standard solution. Plasma samples were 10 times diluted in 99.8 % acetone. As plasma

contains a variety of substances that are not soluble in acetone, we vortexed the solutions for 1 min to mix the layers thoroughly, centrifuged the samples for 5 min at 1500 g, and collected the supernatant. 1 ppm standard solutions were made with 99.8 % acetone.

We conducted mass spectrometry with Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap instrument (Thermo Scientific, USA) equipped with Dionex Ultomate 3000 RSLCnano HPLC system. Mass spectrometric analyses were performed using a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap instrument mass spectrometer, with ESI interface. INNO C18, 3 uL, 120 A, 2 x 100 mm (Youngjin Biochrom Co., Korea) was used for chromatographic separations. The injection volume was 5 uL and the flow rate 150 uL/min. The mobile phase was performed with isocratic 100% methanol. Total run time was 20 min. Ionization of analytes was carried out using electrospray ionization (ESI). The capillary temperature was maintained at 320 °C, the ion source voltage was set at 3.5 kV and the sheath and Aux gas was set at 30 and 5 units. The average scan time was 0.01 min while the average time to change polarity was 0.02 min. The HCD energy was generally chosen in order to maintain about 30 % abundance of the precursor ion.

Results

The list of carotenoids that were detected in the plasma of magpie nestlings is given in Table 2. Detected carotenoids were ordered as the relative intensity levels (i.e. NL values). Due to the presence of isomers, several peaks were detected for certain molecular masses. In many of these cases, we could not identify the carotenoid molecules. With lutein and astaxanthin, we could infer the presence of these carotenoids in the plasma samples of magpie nestlings through the comparisons with peaks observed from standard solutions.

1. Detection of lutein

The absorbance peaks that were observed at the molecular weight of lutein or zeaxanthin, the isomer of lutein, were the largest in all four plasma samples (only the result from 301B is shown in Fig. 1; all the other samples had similar patterns as 301B), which suggests that lutein or zeaxanthin is the most abundant carotenoid in the plasma of magpie nestlings.

Although the molecular weight of lutein is known to be 568.43, m/z spectrum shows that the lutein standard diluted in acetone had the base peak at m/z = 551.43, with m/z = 568.43 being the second most abundant ion (Fig. 1 top panel). The difference between these two peaks is m/z = 17, which coincides with the weight of a hydroxyl group (-OH). Thus, the characteristic mass spectral ions for lutein standard solution were m/z = 568.43 $[M]^+$ and 551.43 $[M + H - 18]^+$. The reason that the loss of one hydroxyl group formed the most abundant ion can be found from the molecular structure of lutein, having two hydroxyl groups on both ends of the molecule. Both of the dominant ions had the ions with m/z higher by 2, which indicates the formation of ions by the double-bond breakage.

Table 2. Carotenoids that are commonly observed in birds and those that were detected in the plasma of magpie nestlings in this study ("o" denotes detection and "x" denotes no detection).

Detected	Range of NL* values	Types of carotenoids	Molecular formula	Molecular mass	Protonated molecular mass	301B	40W	117Bk	117Y
D	1.68E6 – 2.33E7	Lutein, Zeaxanthin	$C_{40}H_{56}O_2$	568.4275	569.4353	o	o	o	o
D	1.15E5 – 4.02E6	Canary xanthophyll A, 3′-dehydrolutein, 3′-hydoxy-echinenone	$C_{40}H_{54}O_2$	566.4118	567.4197	o	o	o	o
D	1.62E5 – 9.09E5	Canary xanthophyll D, Astaxanthin	$C_{40}H_{52}O_4$	596.386	597.3938	o	o	o	o
D	1.57E5 – 3.97E5	Canary xanthophyll C, Papilioerythrinone, Adonirubin	$C_{40}H_{52}O_3$	580.3911	581.3989	o	o	x	o
D	1.73E5 – 3.85E5	Canary xanthophyll B, Canthaxanthin	$C_{40}H_{52}O_2$	564.3962	565.4040	o	o	x	o
D	1.57E5 – 2.63E5	α-doradexanthin, Adonixanthin	$C_{40}H_{54}O_3$	582.4067	583.4146	o	o	o	o
ND		α-carotene, β-carotene	$C_{40}H_{56}$	536.4377	537.4455	x	x	x	x
ND		Rubixanthin, β-cryptoxanthin, Gazaniaxanthin	$C_{40}H_{56}O$	552.4326	553.4404	x	x	x	x
ND		Rhodoxanthin	$C_{40}H_{50}O_2$	562.3805	563.3884	x	x	x	x
ND		7,8-dihydro-β-cryptoxanthin	$C_{40}H_{58}O_2$	570.4431	571.4150	x	x	x	x
ND		7,8,7′,8′-tetrahydrozeaxanthin	$C_{40}H_{60}O_2$	572.4588	573.4666	x	x	x	x
See text		Echinenone	$C_{40}H_{54}O$	550.4169	551.4169	x	x	x	x
		2',3'-anhydrolutein	$C_{40}H_{55}O$	551.4247	552.4326	x	x	x	x

* 'NL' means normalized intensity level (count per second).

All four plasma of magpie nestlings showed peak absorbance at similar ranges of m/z values as lutein standard solution (Fig. 1). Both in m/z ≈ 551.42 and 568.42, plasma samples contained peaks with slightly longer retention times than lutein standard solution, but the pattern with one main peak at retention time around 2 mins and one minor peak at retention time around 3.9 mins was similar to the retention time pattern of lutein standard. m/z ≈ 551.42 and 552.43 coincide with the protonated molecular masses of echinenone and 2'3'-anhydrolutein respectively. However, considering that absorbance pattern with one major peak at 2 mins and a minor peak at 3.9 mins were similar across these molecular masses, we think the molecule that was detected in these m/z ranges was either lutein or zeaxanthin, with some modification such as the loss of hydroxyl group and additional loss of proton, rather than echinenone or 2'3'-anhydrolutein.

It should be noted that normalized intensity level (NL) detected in four plasma samples differed between these two major ions, with m/z ≈ 551.42 being more abundant (2 – 3.7 times, depending on the sample) than m/z ≈ 568.42 (Table 3). In the standard lutein solution, the former ion was approximately 1.62 times more abundant than the latter (Fig. 1 top panel).

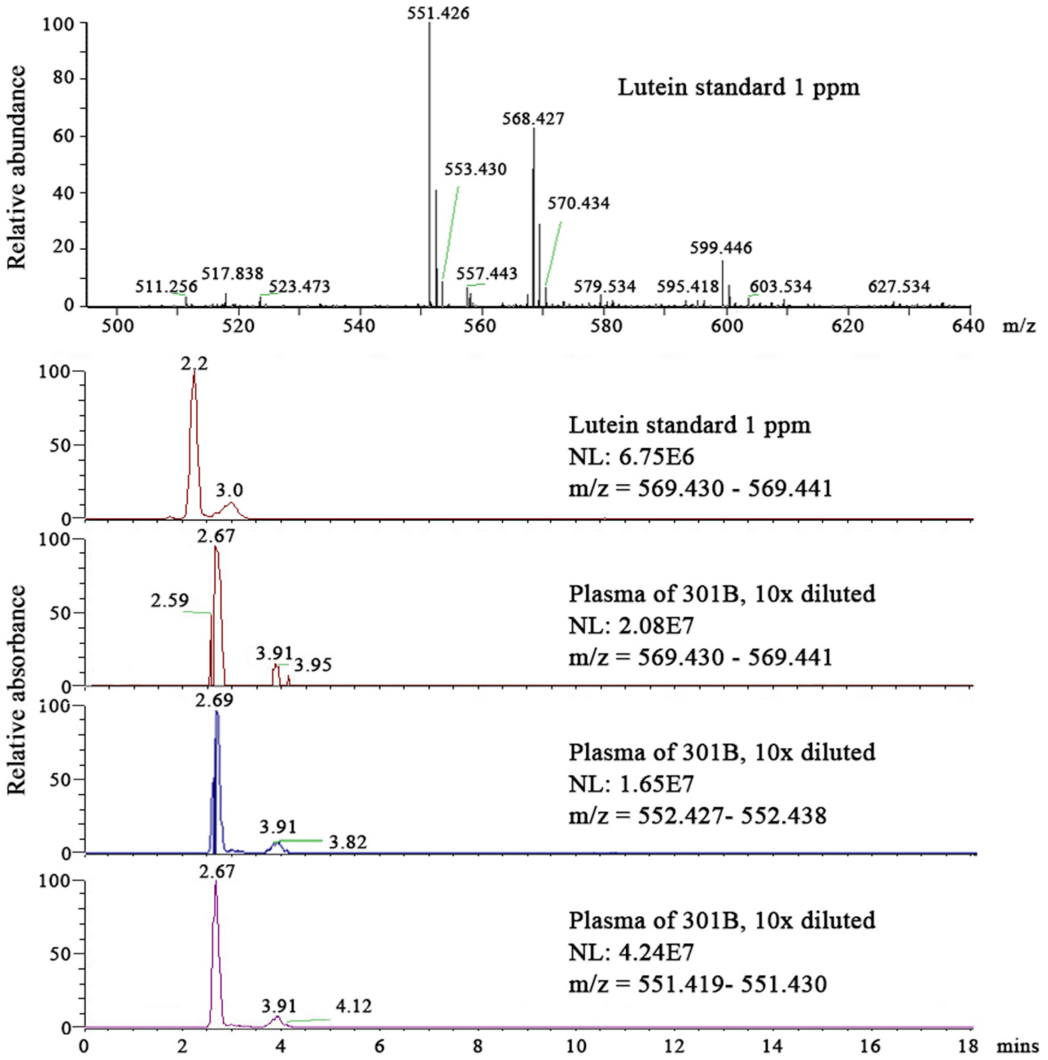

Fig. 1. Detection of lutein in the plasma of magpie nestling 301B. Other samples showed similar patterns. A stick plot obtained from lutein standard suggests that m/z = 551.426 and 568.427 were the most dominant ions (uppermost panel). Lower three panels are the peak absorbance patterns in m/z ≈ 569.43, 552.43 and 551.42. All three panels show the same peak patterns and these are similar to the absorbance peaks of the lutein standard with slight delay in retention time.

Table 3. Comparison of the abundance of two major ions detected in the plasma of magpie nestlings.

ID	NL values		Ratio
	m/z = 569.43	m/z = 551.43	
301B	2.19E7	4.97E7	2.27
40W	2.66E7	5.37E7	2.02
117Bk	4.64E5	9.64E5	2.08
117Y	6.96E6	2.57E7	3.69

2. Detection of astaxanthin

All four plasma samples showed discernible absorbance peaks in the range of molecular weight of astaxanthin (Fig. 2). However, the samples showed slightly different retention times than the standard astaxanthin solution; 301B and 40W showed the peak absorbance at 2.03 and 1.47 mins respectively, whereas standard astaxanthin had the peak absorbance at 1.9 mins (Fig. 2). The other two samples, 117Bk and 117Y had similar absorbance peaks as those of 40W; the main peak was observed at 1.50 and 1.44 mins for 117Bk and 117Y respectively. The reason for the shift is unclear. It might be due to some chemical interaction between various molecules in the plasma of magpie nestlings. It is also possible that one of the two samples contained canary xanthophyll D, the isomer of astaxanthin.

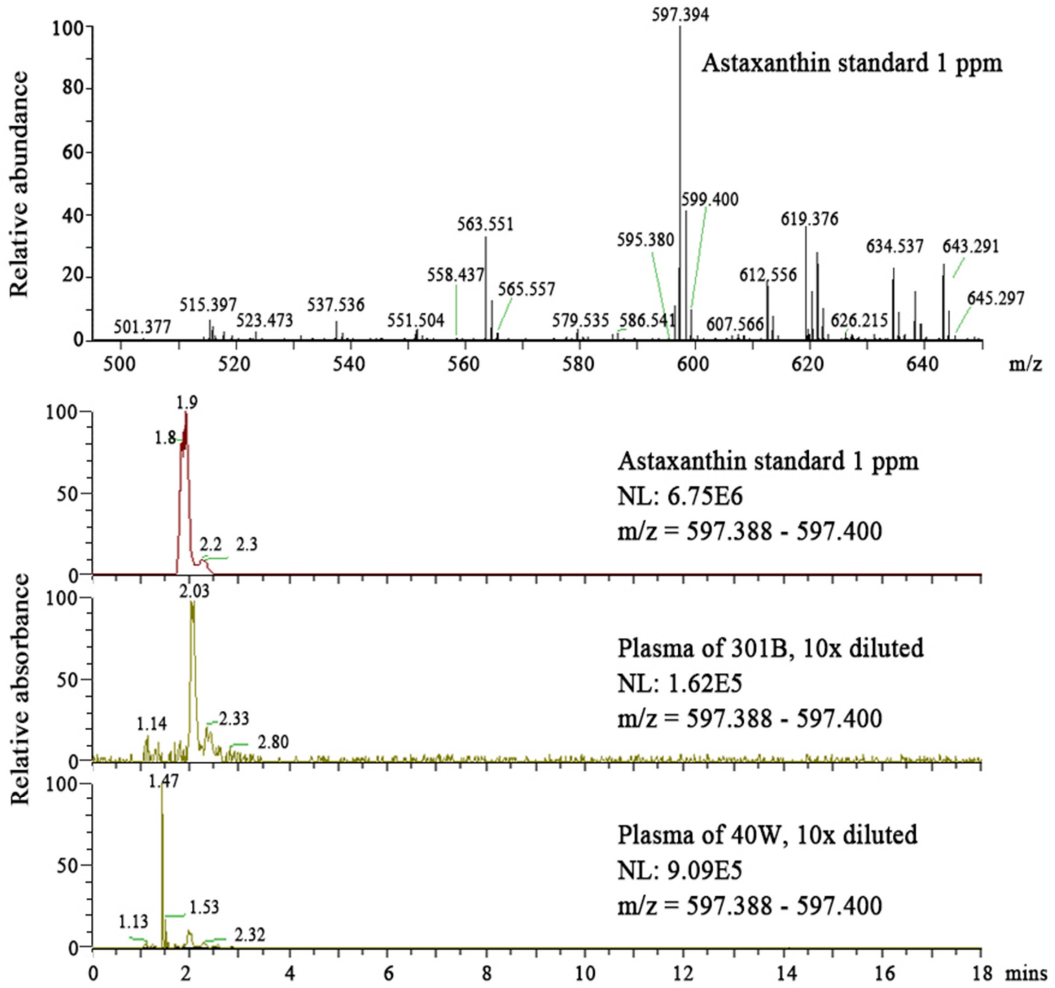

Fig. 2. Detection of astaxanthin in the plasma of magpie nestlings. A stick plot (uppermost panel) obtained from astaxanthin standard suggests that m/z = 597.394 is the most dominant ion. Peaks with similar retention time were observed in the plasma of nestling 301B, whereas peaks with shorter retention time were found in the plasma of nestling 40W.

Discussion

The types of mass spectral ions that lutein forms are consistent to what has been previously reported with lutein diluted in acetone (Sowmya et al. 2014). According to Sowmya et al. (2014), characteristic mass spectral ions of lutein were m/z = 568.8 ($[M]^+$) and 569.8 ($[M + H]^+$). Lutein standard solution used in our study also produced peak in m/z = 568.43 and 569.43 which correspond well to Sowmya et al. (2014)'s result. However, lutein standard solution used in our study produced m/z = 551.43 in even greater abundance than the other two ions. This was similar in all four plasma samples. According to Rivera et al. (2014), fragmentation patterns differed between lutein and zeaxanthin; in the mass spectrum of lutein, the fragment $[M + H - 18]^+$ at m/z = 551 is much more abundant than the one at m/z = 569. In the mass spectrum of zeaxanthin, the isomer of lutein, the opposite is observed. Thus, based on the comparison of the abundance of these two ions, we can guess that the most common carotenoid molecule detected in our samples was lutein rather than zeaxanthin.

It is unclear why there was a shift in retention time in the range of molecular masses of lutein (or zeaxanthin) or its fragmented ions. Based on the literature, it seems that the retention time of zeaxanthin is slightly longer than that of lutein (Heukelem et al. 1994; Huck et al. 2000; McGraw et al. 2002; Deviche et al. 2008). Thus, considering that all four samples showed similar shifts in absorbance peaks towards longer retention time, it is also possible that what has been detected in our study zeaxanthin, rather than lutein. Lutein and zeaxanthin are known to be major plasma carotenoids in some passerines (McGraw 2006). In order to discern which of these two is the major plasma carotenoid in magpie nestling plasma and to verify the specific peak in retention time corresponds to lutein or zeaxanthin, one would include internal standards when running mass spectrometry.

In the molecular mass range of canary xanthophyll D and astaxanthin, we think that it is more likely that what we detected in the magpie nestling plasma is astaxanthin, based on the fact that canary xanthophyll D has been detected only in gold finch among passerines (McDonald 2005).

All the carotenoid that were detected in the plasma of magpie nestlings were xanthophylls. No carotenes were detected. Although lutein and zeaxanthin belongs to the primary dietary carotenoids in birds and serves as the initial substrates for further metabolism and deposition in various tissues (McGraw 2006), they are also the main pigmental constituents of the egg yolk (Rasmussen et al. 2012). Thus, currently we cannot ascertain whether lutein or zeaxanthin in the plasma of magpies have originated from maternal investment through the egg yolk or from the food that was provided after the nestlings hatched. Considering that the two blood samples were taken when the nestlings were around 7 days after hatching and these contained substantial amount of lutein or zeaxanthin, we cannot rule out the possibility that at least some portion of circulating carotenoids in magpie nestlings is attributed to maternal effect.

Some minor constituents of carotenoids in the plasma of magpie nestlings could be regarded as the metabolized forms of lutein or zeaxanthin. Canary xanthophylls A and B and α-doradexanthin are the metabolised forms of lutein. On the other hand, adonixanthin, astaxanthin and 3-dehydro lutein are the metabolized form of zeaxanthin, an isomer of lutein. Although our current analysis does not verify the presence of zeaxanthin, it is possible that these metabolised forms are present in circulating blood in order to be delivered

to be deposited in the tissues where they are utilized. For instance, astaxanthin was the most dominant carotenoid in the retina of the jungle crow (*Corvus macrorhynchos*), another corvid species, as the form of oil droplets (Rahman et al. 2010).

Interestingly, the plasma of magpie nestlings contained both yellow carotenoids such as lutein and zeaxanthin and red carotenoids such as astaxanthin. This may result in the varying degrees of redness in the plasma among the individuals (Lee S-I, pers. obs). Whether the proportion of red to yellow carotenoids cause the colour variation in the plasma among the individuals could be verified by quantifying different carotenoid molecules and correlating the profile with the colour with many plasma samples.

This study was conducted as a preliminary analysis and the main purpose was to find out the major carotenoid molecule in the plasma of magpie nestlings. Since we examined only four nestlings (two of them being siblings and the other two being collected in a different year), we cannot make any meaningful ecological comparisons (e.g. whether siblings or the nestlings produced in the same year share similar profile of carotenoid content). However, considering that all four plasma samples with different ecological backgrounds contained the greatest amount of the molecule whose weight corresponds to that of lutein or zeaxanthin, we can assume that the major carotenoid molecule in the plasma of magpie nestlings is lutein or zeaxanthin. Future studies should discern which of these isomers is the major carotenoid molecule by including internal standards for lutein or zeaxanthin in the mass spectrometry analysis, and include more samples so that ecological comparisons can be made.

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