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ORIGINAL PAPER

Astaxanthin and papilioerythrinone in the skin of birds: a chromatic convergence of two metabolic routes with different precursors?

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Abstract Carotenoids are organic pigments involved in several important physiological functions and may serve as indicators of individual quality in animals. These pigments are only obtained by animals from the diet, but they can be later transformed into other carotenoids by specific enzymatic reactions. The diet of farm-reared and probably wild red-legged partridges (*Alectoris rufa*) is mainly based on cereals that contain high levels of lutein and zeaxanthin. These two carotenoids are also predominant in internal tissues and blood of red-legged partridges. However, in their integuments, astaxanthin and papilioerythrinone (the last one identified in this work) are mainly present in their free form and esterified with fatty acids. According to available literature about carotenoid metabolism in animals, we propose that astaxanthin (λ_{max}=478 nm) and papilioerythrinone (λ_{max}=452–478 nm) are the result of a chromatic convergence of the transformation of dietary zeaxanthin and lutein, respectively. Moreover, the results obtained in this work provide the first identification by liquid chromatography coupled to accurate mass quadrupole time-of-flight mass spectrometer system of papilioerythrinone (*m/z* 581.3989 $[M+H]^+$) in the skin (i.e., not feathers) of a vertebrate. Astaxanthin and papilioerythrinone are very close in terms of chemical structure and coloration, and the combination of these two keto-carotenoids is responsible for the red color of the ornaments in red-legged partridges.

Keywords Carotenoid metabolism · Bird coloration · *Alectoris rufa* · Astaxanthin · Papilioerythrinone · QTOF-MS/MS

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Introduction

Carotenoids are organic pigments that are found naturally in a wide range of living organisms. Over 600 carotenoids have been described in nature (Goodwin 1984; Britton et al. 1995), but this number is still rising due to the identification of new carotenoids in different organisms as algae (Mangoni et al. 2011), fruits (Murillo et al. 2011), animals (Maoka 2009, 2011; LaFountain et al. 2013), among others. In particular, the identification of carotenoids in the external tissues of animals has attracted the attention of zoologists and evolutionary biologists. Animals do not only use carotenoids as pigments, but also as antioxidants (Miller et al. 1996; Stahl and Sies 2003) and immune boosters (Lozano 1994; Chew and Park 2004). However, the only source of these compounds is the diet (Surai 2002; McGraw 2006). This makes individuals producing colorful ornaments face a health-versus-color trade-off for the allocation of probably scarce carotenoids. This trade-off would have favored the evolution of carotenoid-based ornaments as reliable signals of individual quality throughout sexual selection (Lozano 1994; Alonso-Alvarez et al. 2008; Pérez-Rodriguez 2009). As mentioned above, animals incorporate carotenoids from the diet, but these pigments can subsequently be transformed in other diverse carotenoids through specific enzymatic reactions

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(e.g., McGraw 2006; Hill and Johnson 2012). Several metabolic routes have been described in animal species that transform dietary carotenoids into those deposited in integuments (feathers and skin of bare parts in birds; e.g., LaFountain et al. 2013; McGraw 2006; Møller et al. 2000; Stradi et al. 2001).

The red-legged partridge (*Alectoris rufa*) is an important gamebird with high economic and socio-cultural value in Spain (e.g., Gortazar et al. 2000; Arroyo et al. 2012; Díaz-Fernández et al. 2013). This bird has been used as a model species in behavioral and evolutionary biology studies in recent years (e.g., Alonso-Alvarez et al. 2008; Pérez-Rodriguez and Viñuela 2008; Alonso-Alvarez and Galván 2011). In terms of carotenoid identification, García-de Blas et al. (2011) reported that the two main carotenoids responsible of red coloration in the integuments of red-legged partridge were astaxanthin (λ_{max}=478 nm; *m/z* 597.3 $[M+H]^+$) and another red-colored carotenoid (λ_{max}=452–478 nm; *m/z* 581.2 $[M+H]^+$). Combining the information from UV–Vis and MS detectors, García-de Blas et al. (2011) determined that these two carotenoids were present in both free form and esterified with fatty acids. Astaxanthin formed mono- and diesters, whereas carotenoid X only formed monoesters, which suggests that carotenoid X has only one hydroxyl group. The lack of one oxygen atom explains the difference in *m/z* observed. This property coincides with papilioerythrinone ($C_{40}H_{52}O_3$), a ketocarotenoid described in the plumage of some birds (LaFountain et al. 2013; Stradi et al. 2001), insects (Harashima et al. 1972 and 1976), and crustacea (Harashima et al. 1976; Matsuno and Maoka 1988). Despite of the similarity between astaxanthin and papilioerythrinone, these two ketocarotenoids result from the transformation of different dietary precursors (i.e., zeaxanthin and lutein, respectively) (McGraw 2006). Interestingly, astaxanthin in red-legged partridge has been found to be the main carotenoid in both wild and farm-reared red-legged partridges (García-de Blas et al. 2013), whereas zeaxanthin (its precursor) is usually scarcer in plants than lutein (Goodwin 1984).

In this study we identify unequivocally "carotenoid X" as papilioerythrinone. This would be the first time that this carotenoid is identified in the skin (not feathers) of a vertebrate. We used advanced analytical techniques and considered the probable metabolic routes. In this order, comparisons of retention times and spectra (both UV–Vis and MS) of all pigments of mass 580.3 Da were performed. This was made (1) on the basis of available literature, (2) by using commercial standards or (3) by the extraction of carotenoids from its natural sources. Different chromatographic and detection techniques such as high performance liquid chromatography coupled to diode array detector (HPLC-DAD), mass spectrometry (HPLC-MS)and tandem mass spectrometry (LC-MS/MS) detectors were applied. Here we discuss the possibility that papilioerythrinone could be the best alternative for red-legged partridges to obtain a similar red color such as that produced by astaxanthin, especially because the precursor of the former is more abundant.

Material and methods

Reagents

Hexane, *tert*-butyl methyl ether (TBME), methanol (MeOH), and acetone were purchased from Prolabo (VWR, Lutterworth, UK). All chemicals were HPLC gradient grade. Carotenoid standards of lutein, zeaxanthin, astaxanthin, astaxanthin dipalmitate and adonirubin (phoenicoxanthin/3-hydroxycanthaxanthin) were purchased from CaroteNature (Lupsingen, Switzerland). Non-commercially available carotenoids (pectenolone and papiliorerythrinone) were obtained from natural sources to use these extracts as standards.

Extraction of carotenoids

Carotenoid X was extracted from bare parts (i.e., eye rings, bill and legs) of a sample of red-legged partridges (n=30) following the method described by García-de Blas et al. (2013).

Pectenolone was extracted from the gonad of giant scallops *Pecten maximus* (n=4) purchased from a food market in Spain. The extraction procedure was based on Suhnel et al. (2009) with few modifications. A scallop sample (500 mg) was transferred to a 15 ml tube and homogenized with 10 ml of solvent mixture (acetone/hexane, 1:3 [v/v]), and then it was shaken vigorously during 15 min and centrifuged (2,026×g, 5 min). The supernatant was evaporated to dryness under N_2 stream.

Papilioerythrinone was extracted from the entire orange pupae (n=5) of swallowtail butterflies (*Papilio xuthus*) (Harashima et al. 1972), the whole of the carapace of one Hanasakigani crab (*Paralithodes brevipes*) (Harashima et al. 1976; Matsuno and Maoka 1988) and breast feathers of male (n=23) common bullfinches (*Pyrrhula pyrrhula*) (Stradi 1998; Stradi et al. 2001). Pupae of swallowtail and crab carapaces were kindly provided by Akira Yamanaka (Department of Biology and Chemistry, Yamaguchi University, Japan) and Sayuri Shigematsu (Department of Microbiology and Immunology, Nagasaki University, Japan), respectively. Pupae integuments and crab carapaces were washed with 0.5 ml of NaCl and stirred vigorously for 1 min. Then, carotenoids were extracted with 2 ml of acetone in an electric homogenizer (Ultra-turrax VDI 12, VWR, Barcelona, Spain) for 30 s. This step was carried out twice. The sample was kept on ice avoiding exposure to light. The two pooled extracts were centrifuged (2,026×g, 5 min) and supernatants were mixed with 2 ml of hexane and they were stirred vigorously

for 1 min. Pooled extract was centrifuged again (2,026×*g*, 5 min), and the supernatant was evaporated to dryness under N_2 stream. Bullfinch feathers were provided by the Estación Biológica de Doñana (CSIC, Sevilla, Spain) and the Museo Nacional de Ciencias Naturales (CSIC, Madrid, Spain). These feathers were analyzed following the method described by McGraw and Hardy (2006), with some modifications. The red portion of the feathers was washed with ethanol and hexane to remove the lipid component of the feathers, and then it was extracted in a glass tube (with N_2 in the headspace) with acidified pyridine at 95 °C in a water bath, for 15 min. Pyridine was acidified with a few HCl drops. After this, it was cooled down to room temperature and 5 ml of water were added. The tube was inverted several times and then 2.5 ml of hexane/TBME (1:1 [v/v]) were added. It was stirred vigorously for 2 min and centrifuged (2,026×*g* for 5 min). The supernatant was evaporated under N_2 stream.

Dry extracts of carotenoids obtained from all these natural sources were re-dissolved in appropriate amount of the mobile phase described below and analyzed by HPLC techniques.

Instrumentation for carotenoid analyses

Two LC systems were used in this work. An Agilent 1100 Series system equipped with a photodiode array detector (DAD) online with a single quadrupole mass spectrometer (MS) (Agilent 6110 LC/MS Quadrupole) was used. In order to obtain accurate mass and MS/MS data, an Agilent 1260 Infinity LC system coupled to a quadrupole-time of flight (Q-TOF) mass spectrometer (Agilent 6530 Q-TOF MS) was also used. In both cases, the analyses were carried out following the method described by García-de Blas et al. (2011). Briefly, the column was specific for analysis of carotenoids (YMC-30 Carotenoid 5 μm, 250×4.6 mm id). The mobile phase was MeOH/TBME/H_2O (81:15:4) (phase A) and TBME/MeOH (90:10) (phase B). The initial chromatographic conditions were 99 % of A and 1 % B, with a first gradient change to 44 % A and 56 % B in 78 min, a second gradient to 100 % B in 12 min, and then to return to initial conditions in 10 min, followed by a stabilization time under these conditions for 10 min before the next run. The injection volume was 20 μl and the flow rate was 0.5 ml/min.

The DAD scan range was 300–600 nm, but the detection and quantification was carried out at 480 nm. The determination by LC-MS was carried out using positive-ion by Atmospheric-Pressure Chemical Ionization (APCI), sweeping mass of 100 to 620 *m/z*. The drying gas used was N_2 at a flow of 10 l/min, nebulizer pressure was set at 45 psi, the drying gas temperature was 320 °C, vaporizer temperature was 325 °C, capillary voltage was set at 3,500 V, crown intensity was 5 μA and the charging voltage of 2,000 V and shredder to 100 V. The analysis by LC-MS/MS was performed with an electrospray ionization source in positive mode. During all analysis two reference masses were used: *m/z* 121.0509 ($C_5H_4N_4$) and *m/z* 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$). These masses were continuously infused to the system to allow constant mass correction. Targeted MS/MS were carried out at 20 V collision energy over the 581.3989 *m/z* precursor ion $(C_{40}H_{52}O_3+H)^+$ at a retention time of 18.5±2.0 min with the same settings as in LC-MS analyses. For system control and data acquisition in the LC-MS/MS analysis the Agilent MassHunter Acquisition software was used. Data reprocessing was performed by Agilent MassHunter Qualitative Analysis Software and the study of the fragmentation of MS/MS was carried out by using Agilent's Molecular Structure Correlator software.

Results

Identification of carotenoid X: screening by structural information, chromatographic behavior and UV–Vis spectra

The chromatogram with DAD detection of the red-legged partridge integuments shows the presence of carotenoid X at the retention time of 20.927 min (Fig. 1). The swallowtail pupae (Fig. 2a) and the carapace of Hanasakigani crab (Fig. 2b) did not show any major carotenoid with the same retention time and UV–Vis spectrum as carotenoid X (Fig. 1). However, in the pupae, papilioerythrinone shows as a minor peak that indeed matches the retention time and UV–Vis spectrum of carotenoid X, though it was too small to have a good definition in the spectrum. In the extract of the bullfinch feathers we have observed the corresponding peak to papilioerythrinone at 20.886 min, with this flattened UV–Vis spectrum (Fig. 2c), and a similar retention time (20.927 min) and spectrum was observed for carotenoid X from the extracts of integuments of red-legged partridges (Fig. 1).

Identification of papilioerythrinone by mass spectrometry

The accurate masses ($[M+H]^+$) obtained by LC-QTOF-MS/MS of the peak identified as papilioerythrinone in common bullfinch feathers and the carotenoid X peak in red-legged partridge integuments were *m/z* 581.3998 and 581.3993, respectively. In the mass spectrum of these peaks, there was also an ion at *m/z* 603.3826 in bullfinches and at *m/z* 603.3823 in partridges, which may correspond to $[M+Na]^+$. Papilioerythrinone in bullfinches and carotenoid X showed the same isotopic distribution for the molecular ion (Fig. 3) and fragmentation pattern in MS/MS spectra (Fig. 4). Theoretical exact mass calculated for the molecular ion of papilioerythrinone ($C_{40}H_{53}O_3$; $[M+H]^+$) was 581.3989. The difference of this mass with respect to exact mass calculated for carotenoid X of red-legged partridges (581.3993) was −0.72 ppm and for papilioerythrinone of bullfinch feathers

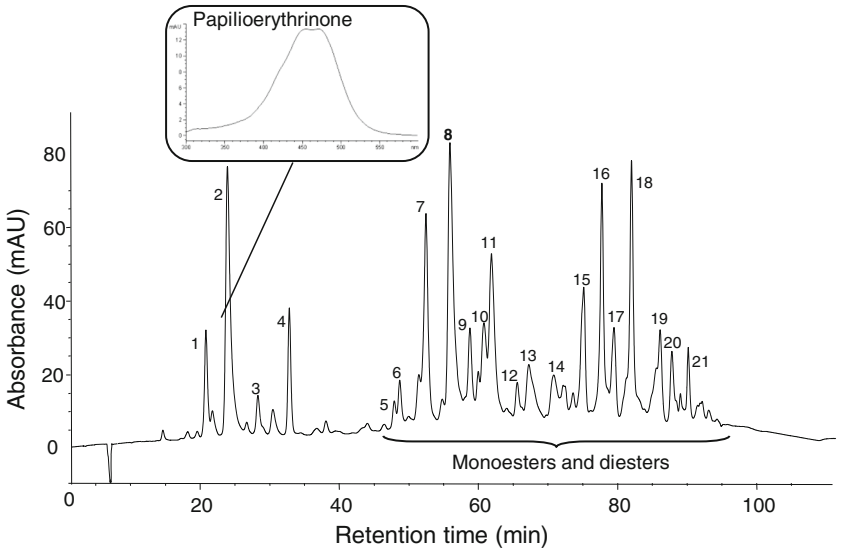


Fig. 1 Chromatogram (HPLC-DAD) of the integuments of red legged partridges. **1** Carotenoid X (identified as papilioerythrinone in this work), **2** astaxanthin, **3** lutein, **4** cantaxanthin; UV–Vis spectrum of papilioerythrinone is included, too. Astaxanthin and papilioerythrinone esters were identified on basis to their UV–Vis and MS spectra (described by García-de Blas et al. 2011). Monoesters of papilioerythrinone: **5** papilioerythrinone monolinoleate, **7** papilioerythrinone monooleate, **9** papilioerythrinone palmitate, **12** monoester not identified. Monoesters of astaxanthin: **6** astaxanthin monolinoleate, **8** astaxanthin monooleate, **10** monoester not identified, **11** astaxanthin monopalmitate, **13** monoester not identified. Numbers **14** to **21** are astaxanthin diesters (in this case, the fatty acids forming diesters were not identified, only astaxanthin dipalmitate (**19**), which was identified by comparison with the commercial standard)

(581.3998) was −1.48 ppm (Fig. 3). Besides, the relative abundance of ions forming molecular clusters is the same in carotenoid X and papilioerythrinone. These values are shown in the table contained in Fig. 3. For the sodium adduct of papilioerythrinone ($C_{40}H_{52}O_3Na$; $[M+Na]^+$), theoretical exact mass calculated was 603.3814; in this case, the difference of this mass with respect to mass calculated for corresponding observed ion of carotenoid X of red-legged partridges (603.3823) was −1.49 ppm and for papilioerythrinone of bullfinch feathers (603.3826) was −1.98 ppm.

MS and MS/MS spectra of astaxanthin standard were also obtained for comparison with the papilioerythrinone extracted from bullfinches (and carotenoid X from partridges) because both carotenoids have similar chemical structure, and hence, they should provide similar fragmentation patterns (Fig. 5). The main ions observed were:

- Astaxanthin: $[M+Na]^+$ (*m/z* 619.8759); $[M+H]^+$ (*m/z* 597.3917); $[M+Na\text{-}18]^+$ (*m/z* 601.3585); $[M+Na\text{-}92]^+$ (*m/z* 527.2853); $[M+Na\text{-}106]^+$ (*m/z* 513.413); $[M+Na\text{-}138]^+$ (*m/z* 481.2629).
- Papilioerythrinone: $[M+Na]^+$ (*m/z* 603.3826); $[M+H]^+$ (*m/z* 581.3998); $[M+Na\text{-}18]^+$ (*m/z* 585.2952); $[M+Na\text{-}92]^+$ (*m/z* 511.3187); $[M+Na\text{-}106]^+$ (*m/z* 497.3049); $[M+Na\text{-}138]^+$ (*m/z* 465.2775).
- Carotenoid X: $[M+Na]^+$ (*m/z* 603.3823); $[M+H]^+$ (*m/z* 581.3993); $[M+Na\text{-}18]^+$ (*m/z* 585.2887); $[M+Na\text{-}92]^+$ (*m/z* 511.3194); $[M+Na\text{-}106]^+$ (*m/z* 497.3036); $[M+Na\text{-}138]^+$ (*m/z* 465.2803).

Discussion

Our results as a whole allowed identifying the second major carotenoid present in red-legged partridges ornaments ("carotenoid X"). We considered and subsequently discarded those carotenoids with structure like astaxanthin and mass of 580.3 Da, i.e., pectenolone, (4R)-4-hydroxyalloxanthin, methoxy-keto-micoxanthin, 4-(D-glucopyranosyloxy)-7′,8′-dihydro-4,4′-diaponeurosporene, 2-hydroxycantaxanthin and adonirubin (www.lipidbank.jp). We initially discarded 4-(D-glucopyranosyloxy)-7′,8′-dihydro-4,4′-diaponeurosporene, methoxy-keto-micoxanthin and (4*R*)-4-hydroxyalloxanthin ((3*S*,4*R*,3′*R*)-4-hydroxyalloxanthin) according to the available information in the literature because none of them show a structure similar to astaxanthin (Britton 1995) or meet the necessary requirements to form monoesters only, this is that they contain a single hydroxyl group or present a second hydroxyl radical less capable of forming esters (García-de Blas et al. 2011). Adonirubin was discarded because the retention time (30.663 min) and the UV–Vis spectrum (λ_{max}=477 nm) that we obtained from the standard did not match the data obtained for carotenoid X (see Online resource 1). Since 2-hydroxycanthaxanthin is just a positional isomer of adonirubin, it was discarded because 2-hydroxy-canthaxantin probably presents a retention time and UV spectrum similar to adonirrubin (also 3-hydroxy-cantaxanthin). Similarly, none of the chromatographic peaks obtained from the giant scallop that may correspond to pectenolone had the UV–Vis spectrum of carotenoid X (See online resource 2).

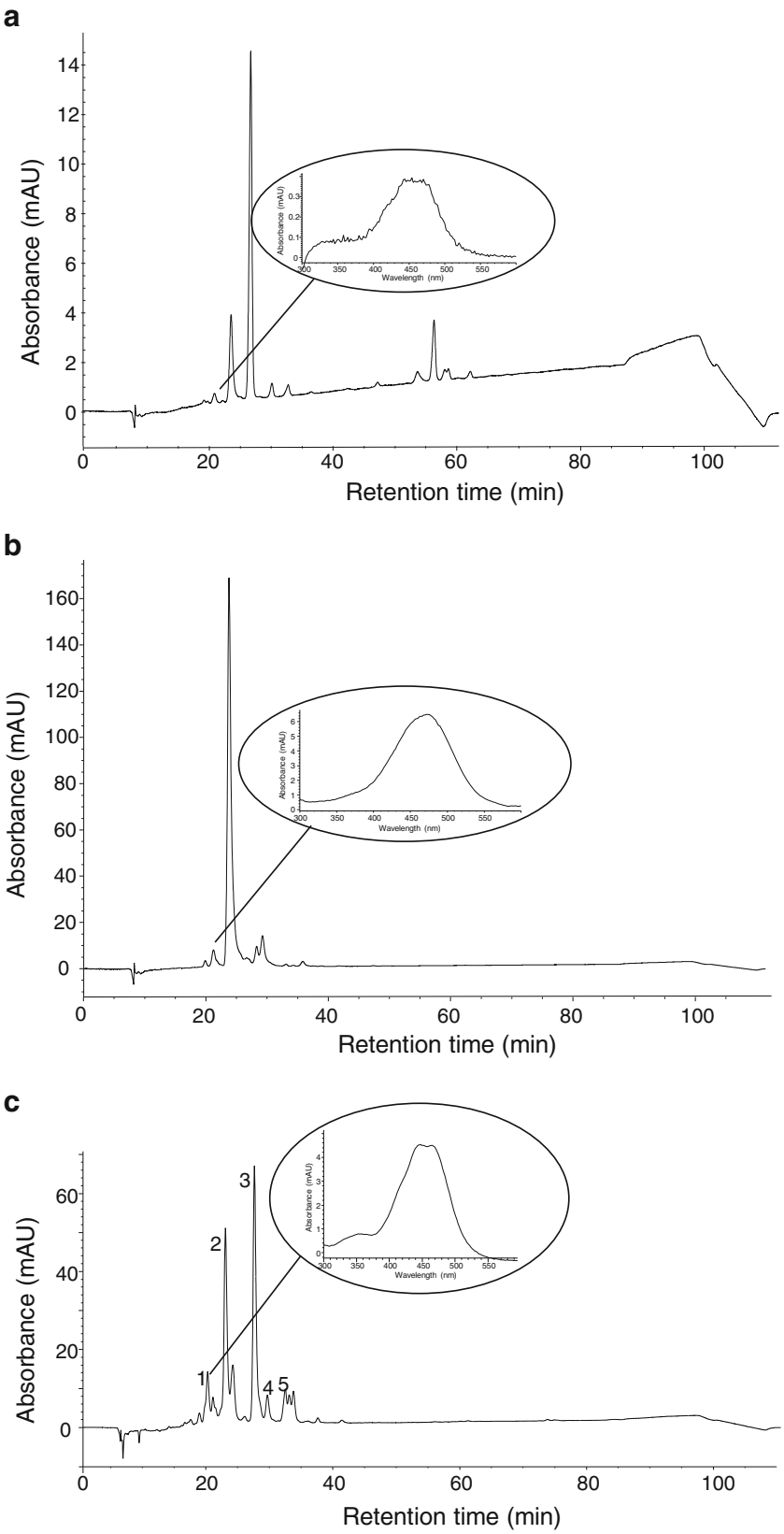
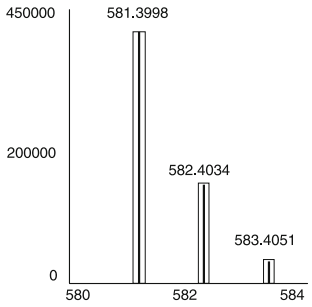


Fig. 2 Chromatogram (HPLC-DAD) corresponding to the entire orange pupae of a swallowtail (**a**), carapaces of a Japanese crab (**b**) and feathers of common bullfinch feathers (c). It has not been possible to identify carotenoids in **a** and **b** due to the absence of commercial standards. **c** **1** Papilioerythrinone, **2** astaxanthin, **3** adonirubin, **4** α-doradexanthin, **5** cantaxanthin (identification as Stradi et al. 2001); UV–Vis spectra of papilioerythrinone and carotenoids at the same retention time are also included

The fact that swallowtail pupae and the carapace of Hanasakigani crab did not show any major carotenoid with the same retention time and UV–Vis spectrum as carotenoid X, is in agreement with Harashima et al. (1972, 1976) that

Height % (Calc.)	m/z (Calc.)	Height % (Papilioerythrinone)	m/z (Papilioerythrinone)	Diff (ppm)
100	581.3989	100	581.3998	-1.48
44	582.4023	45.7	582.4034	-1.81
10.1	583.4056	11.8	583.4051	0.72



Height % (Calc.)	m/z (Calc.)	Height % (Carotenoid X)	m/z (Carotenoid X)	Diff (ppm)
100	581.3989	100	581.3993	-0.72
44	582.4023	41.6	582.4024	-0.16
10.1	583.4056	10.4	583.404	2.75

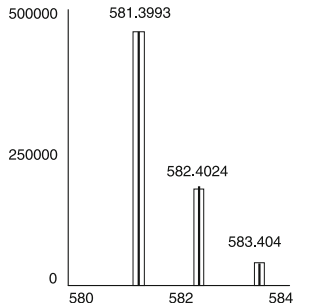


Fig. 3 Isotopic pattern of molecular ions of papilioerythrinone and carotenoid X

performed the identification of papilioerythrinone in pupae and crabs by column chromatography and thin layer chromatography. They also described that the main carotenoid present in these samples was papilioerythrin (identical to α-doradexanthin: $C_{40}H_{54}O_3$), papilioerythrinone being only a minor carotenoid in both organisms.

On the other hand, we have observed a peak in the extract of the feathers of male common bullfinch showing the same retention time and UV–Vis spectra than the carotenoid X of red-legged partridge. Stradi et al. (2001) identified astaxanthin, α-doradexanthin, adonirubin, cantaxanthin and papilioerythrinone in breast feathers of male common bullfinch. They also described an absorption spectrum for papilioerythrinone with a flattened shape at the crest and λ_{max} at 452–478 nm. These findings strongly suggest that carotenoid X is the same molecule that Stradi et al. (2001) identified as papilioerythrinone in male bullfinch feathers.

We have also observed cantaxanthin in the integuments of farm-reared red-legged partridges (Fig. 1), because this keto-carotenoid is usually supplemented in the commercial feed used for this species (García-de Blas et al. 2011). However, wild red-legged partridges tend to have much lower values than farm-reared birds (García-de Blas et al. 2013).

The results obtained from LC-QTOF-MS/MS show the typical ions of the fragmentation of carotenoids (Britton et al. 1995) and they are in agreement with to fragmentation reported in the literature (Britton et al. 1995; Enzell et al. 1968; Enzell and Back 1995). The identification of carotenoid X as the same molecule found in male bullfinch feathers was reinforced with the Molecular Structure Correlator (MSC) software, which compares the obtained MS/MS spectra (i.e., carotenoid X) with the calculated theoretically from known molecules (here papilioerythrinone). In this case, MSC provided a list of ions and elucidated 70 % of them with 99.9 % weight (See online resource 3).

The presence of papilioerythrinone has been reported in feathers of some passerine birds (Stradi et al. 2001; LaFountain et al. 2013), but this work is the first in which papilioerythrinone is detected in the skin (not feathers) of any vertebrate, and also the first time for any tissue in non-passerine avian species. Furthermore, our previous studies are the first in showing that papilioerythrinone can be esterified with different fatty acids in animal integuments, which makes carotenoids more stable against oxidation (see García-de Blas et al. 2013).

Regarding metabolic transformations of ingested carotenoids in red-legged partridge, astaxanthin and papilioerythrinone are the main carotenoids present in their integuments, but neither their commercial diet (García-de Blas et al. 2011) nor the liver and plasma of partridges with the same diet contain these two keto-carotenoids (Rodríguez-Estival et al. 2010). This indicates the possibility of a transformation of dietary zeaxanthin and lutein into astaxanthin and papilioerythrinone in the integument, as previously suggested for other birds (McGraw 2006). Stradi et al. (2001) and LaFountain et al. (2013), among others, proposed that papilioerythrinone is the result of transformation of dietary carotenoids, and more specifically, of lutein (Fig. 6). They suggested that lutein is oxidized to an intermediary compound (papilioerythrin [α-doradexanthin: (3*S*,3′*S*,6′*R*)-3,3′-dihydroxy-β-ε-caroten-4-one] or fritschiellaxanthin ((3*S*,3′*R*,6′*R*)-3,3′-dihydroxy-β-ε-caroten-4-one), and this one is later transformed to papilioerythrinone ((3*S*,6′*R*)-3-hydroxy-β-ε-carotene-4,3′-dione) after dehydrogenation (Fig. 6). In the same way, zeaxanthin is transformed to astaxanthin after two 4-oxidations of the two β-end groups. Zeaxanthin ((3*R*,3′*R*)-β-β-carotene-3,3′-diol) is oxidized first to adonixanthin (4-keto-zeaxanthin: (3*S*,3′*R*)-3,3′-dihydroxy-β-β-caroten-4-one) and this one later leads to astaxanthin (3*S*,3′*S*)-3,3′-dihydroxy-β-β-caroten-4,4′-dione) (Møller et al. 2000; Lemoine and Schoefs 2010; LaFountain et al. 2013) (Fig. 6). Nonetheless, we have not found α-doradexanthin (neither fritschiellaxanthin) or adonixanthin in the integument of the red-legged partridge. To explain this, we may tentatively propose a high efficacy of those unknown

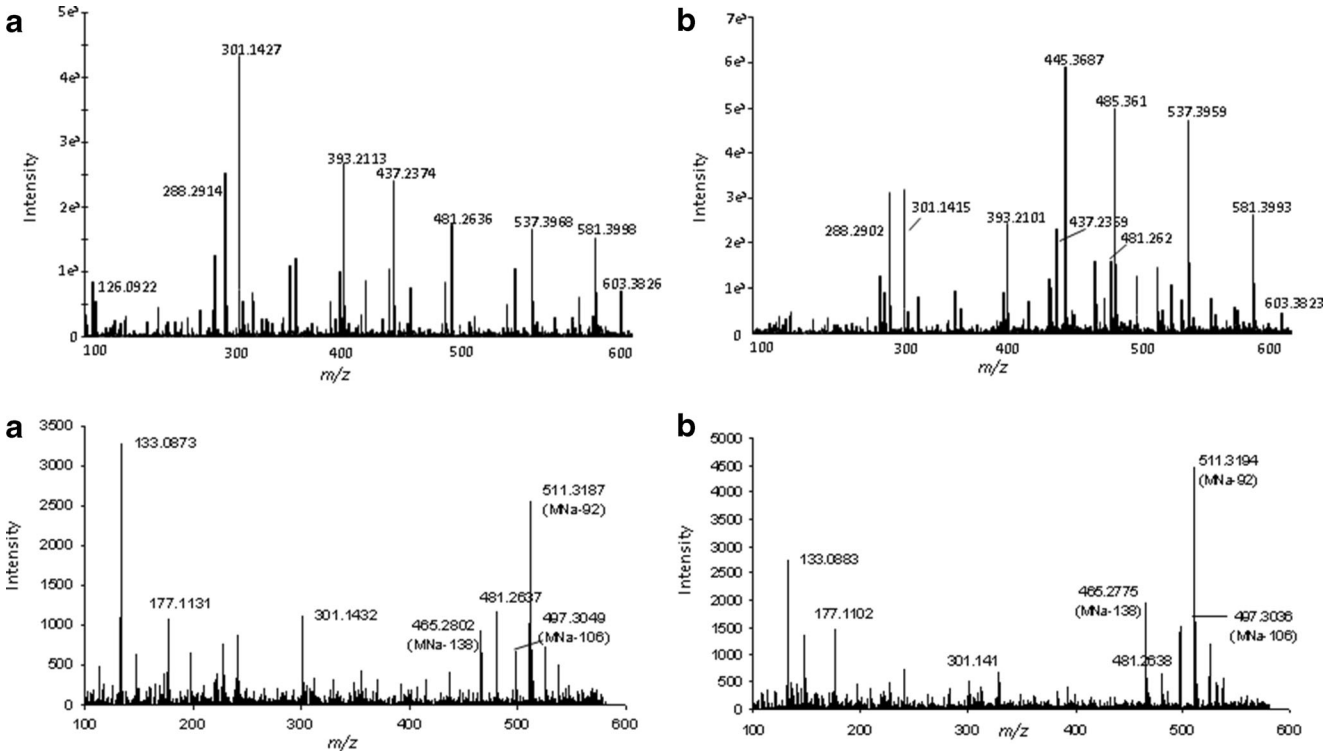


Fig. 4 MS (*top*) and MS–MS (*bottom*) spectra of papilioerythrinone (**a**) and carotenoid X (**b**). Precursor ion is 581.3989 at a retention time of 18.5± 2 min in both cases

enzymatic routes involved in these biotransformations, perhaps involving simultaneous transformations, and hence preventing to detect intermediate compounds (Hill and Johnson 2012). These enzymes are still unknown (e.g., Hill and Johnson 2012). However, in the case of papilioerythrinone, we could mention that the biochemical characteristics of the candidate 4-oxidase and dehydrogenase suggest that they should occupy opposite sides of the bilayer cell membrane (James D. Johnson, personal communication), allowing a quick biotransformation of the lutein substrate.

It is known that birds have selective capacities to accumulate different carotenoids in their tissues in order to obtain brightest colors (McGraw 2006). These strategies of coloration, which are favored by sexual selection, are related to available quantities of circulating carotenoid precursors (McGraw and Gregory 2004). Frequently, red to yellow plumages are the result of the combination of several types of carotenoids with different color (some yellow and some red pigments, (McGraw 2006)). Interestingly, our work has shown that the red integuments of red-legged partridges are

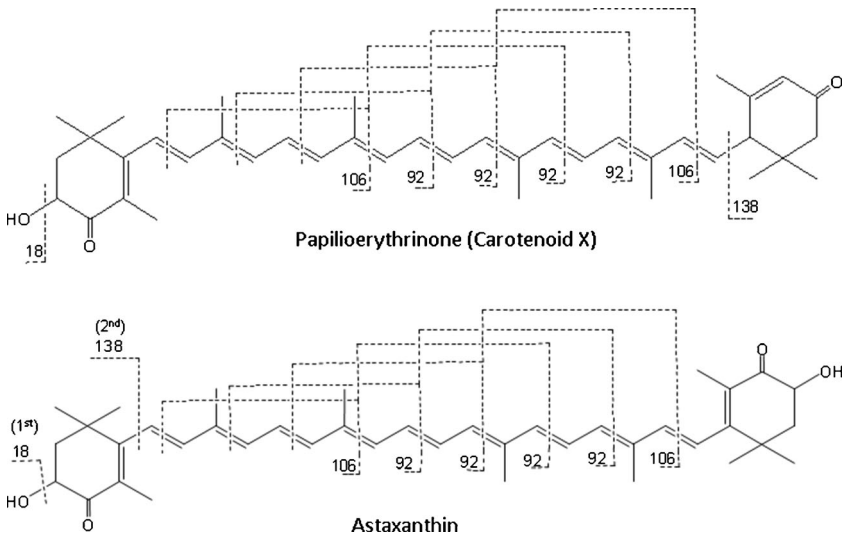


Fig. 5 Propose fragmentation of papilioerythrinone (carotenoid X) and astaxanthin under MS/MS conditions

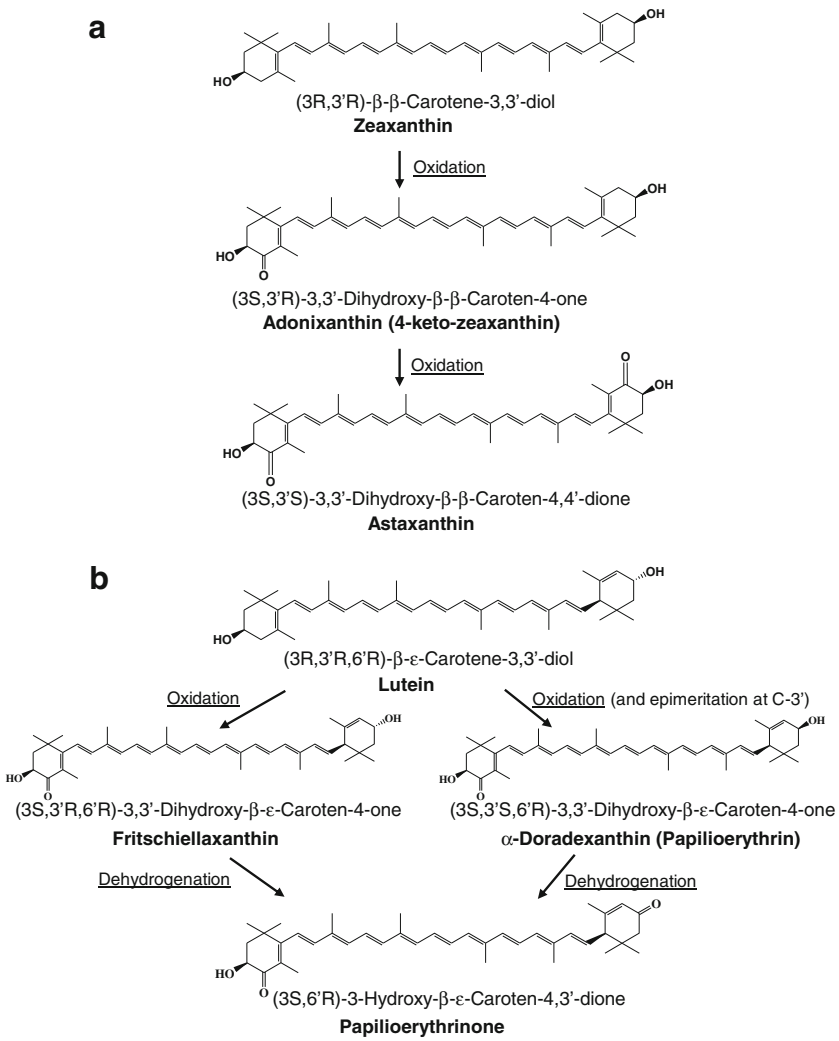


Fig. 6 Probable metabolic route of transformation of dietary carotenoids into integument carotenoids in red-legged partridges. **a** From zeaxanthin to astaxanthin. **b** From lutein to papilioerythrinone. Metabolic pathways have been proposed on basis to McGraw (2006, and references cited therein) and LaFountain et al. (2013)

created by two keto-carotenoids that provide a very similar red color, despite of being synthesized from different dietary precursors.

Both astaxanthin and papilioerythrinone are redder than their intermediate compounds. Adonixanthin, which is the intermediate product of the zeaxanthin–astaxanthin pathway, has a λ_{max} of 470 nm (in the mobile phase of the present study). In the alternative lutein–papilioerythrinone route, fritschiellaxanthin has λ_{max} of 450 nm (in petroleum ether; Matsuno and Ookubo 1982) and α-doradexanthin has λ_{max} of 453–469 nm (in petroleum ether; Castillo and Lenel 1978). However, both metabolic routes have yielded red ketocarotenoids showing very similar λ_{max} values up to 478 nm from the yellow hydroxycarotenoids zeaxanthin (λ_{max}=428–450–476) and lutein (λ_{max}=424–444–472 nm) (both in the mobile phase used here). This can be considered as a sort of chromatic convergence of the two metabolic routes. The result could be that both pigments contribute to the color of bare parts (see García-de Blas et al. 2013), but could perhaps be indistinguishable for the partridges' eyes. Although we do not know the peak of spectral sensitivity of the red-legged partridge photoreceptors, short-wavelength single (SWS) cones in other gallinacean (*Gallus gallus*) show a peak at 475 nm (Hart and Vorobyev 2005; see also Cuthill 2006), which is very close to λ_{max} values of both astaxanthin and papilioerythrinone. Furthermore, we have

Table 1 Composition of carotenoids in integuments (eye rings, bills and legs) of wild red-legged partridges (N=29)

Pigment	Mean±SD
Free astaxanthin	187.9±235.1
Free papilioerythrinone	22.6±31.1
Lutein	4.5±4.1
Cantaxanthin	10.6±10.4
Astaxanthin monoesters	544.5±668.9
Astaxanthin diesters	4913.3±4558.6
Papilioerythrinone monoesters	265.4±316.4

Concentration are expressed in nmol of carotenoid per gram of tissue. See García-de Blas et al. (2011, 2013) for more details

previously shown that manipulation of bill and eye rings of male partridges by means of red paint similar to the original color of the species induced changes in the behavior of their mates, which produced more eggs during the laying period (Alonso-Alvarez et al. 2012).

Most part of variability in the pigments of red-legged partridge integuments is due to astaxanthin diesters (Table 1), which should be created from zeaxanthin. An intriguing question is if the λ_{max} of papilioerythrinone obtained from the most abundant carotenoid in the diet (i.e., lutein) converged with that from astaxanthin, why astaxanthin levels in ornaments are much higher than papilioerythrinone levels (see Table 1 and García-de Blas et al. 2011, 2013)? A potential explanation requires an evolutionary approach. If carotenoid-based ornaments of red-legged partridges are the result of sexual selection (see Alonso-Alvarez et al. 2012), their evolution could depend on the costs associated to their production. Only high quality birds would be able to face these costs, assuring the reliability of the traits as signals of individual quality (the handicap theory; sensu Zahavi 1975; also Grafen 1990). The fact that zeaxanthin is proportionally less abundant in food may imply a cost because animals should increase their food intake. Alternatively or additionally, different metabolic routes could be more or less costly. The astaxanthin–zeaxanthin route (two oxidative steps) could be more sensitive to alterations in the redox state than the papilioerythrinone–lutein pathway (one oxidation and one dehydrogenation), being favored by sexual selection to produce the ornaments of red-legged partridges. Testing these hypotheses will require further correlational and experimental approaches.

Conclusions

Our results confirm therefore, that carotenoid X present in red-legged partridges is papilioerythrinone. This conclusion was reached because papilioerythrinone and carotenoid X have the same retention time and UV–Vis spectrum (Figs. 1 and 2), similar isotopic distributions (Fig. 3) and patterns of fragmentation in MS–MS (Fig. 4). Moreover, we may conclude that papilioerythrinone and astaxanthin are the metabolic results of oxidation of dietary carotenoids (Fig. 6) because neither papilioerythrinone nor astaxanthin were present in the commercial diets of the studied partridges. This raises new questions about the metabolic routes to produce papilioerythrinone and astaxanthin from dietary sources, which may have important implications for understanding the evolution of carotenoid-based ornaments as sexual signals. Finally, the results also emphasize the necessity of identifying those carotenoids giving color to animals before adding them to dietary supplements. Different companies add natural (commonly cantaxanthin) or synthetic carotenoids (usually an ester of β-apo-8-carotenoic acid; e.g., Pérez-Bonilla et al. 2011) to pelleted food in order to intensify the red coloration of these birds. These carotenoids are unrelated to the metabolic routes here described, and it is unknown what effects on color of birds may exert.

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References

Alonso-Alvarez C, Galván I (2011) Free radical exposure creates paler carotenoid-based ornaments: a possible interaction in the expression of black and red traits. PLoS ONE 6:e19403

Alonso-Alvarez C, Pérez-Rodríguez L, Mateo R, Chastel O, Viñuela J (2008) The oxidation handicap hypothesis and the carotenoid allocation trade-off. J Evol Biol 21:1789–1797

Alonso-Alvarez C, Pérez-Rodríguez L, Ferrero ME, García-de Blas E, Casas F, Mougeot F (2012) Adjustment of female reproductive investment according to male carotenoid-based ornamentation in a gallinaceous bird. Behav Ecol Sociobiol 66:731–742

Arroyo B, Delibes-Mateos M, Díaz-Fernández S, Viñuela J (2012) Hunting management in relation to profitability aims: red-legged partridge hunting in central Spain. Eur J Wildl Res 58:847–855

Britton G (1995) Structure and properties of carotenoids in relation to function. FASEB J 9:1551–1558

Britton G, Liaaen-Jensen S, Pfander H (1995) Carotenoids, Volume 1B: Spectroscopy, Birkhäuser Verlag Basel Switzerland

Castillo R, Lenel R (1978) Determination and metabolism of carotenoid pigments in the hermit crab *Clibanarius erythropus* latreille (1818) [Syn. *Cl. misanthropus* heller (1863)]. Comp Biochem Physiol 59: 67–73

Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134:257S–261S

Cuthill I (2006) Color perception. In: Hill GE, McGraw KJ (eds) Bird coloration, vol 1, Mechanisms and measurements. Havard University Press, Cambridge, pp 3–40

Díaz-Fernández S, Arroyo B, Casas F, Martínez-Haro M, Viñuela J (2013) Effect of game management on wild red-legged partridge abundance. PLoS ONE 8:e66671

Enzell CR, Back S (1995) Mass spectrometry. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids, Volume 1B: Spectroscopy. Birkhäuser Verlag, Switzerland, pp 261–320

Enzell CR, Francis GW, Liaaen-Jensen S (1968) Mass spectrometric studies of carotenoids: occurrence and intensity ratios of M-92 and M-106 peaks. Acta Chem Scand 22:1054–1055

García-de Blas E, Mateo R, Viñuela J, Alonso-Alvarez C (2011) Identification of carotenoid pigments and their fatty acid esters in an avian integument combining HPLC-DAD and LC-MS analyses. J Chromatogr B 879:341–348

García-de Blas E, Mateo R, Viñuela J, Pérez-Rodríguez L, Alonso-Alvarez C (2013) Free and esterified carotenoids in ornaments of an avian species: the relationship to color expression and sources of variability. Physiol Biochem Zool 86:483–498

Goodwin TW (1984) The biochemistry of the carotenoids, Volume II: Animals. Chapman and Hall, London

Gortazar C, Villafuerte R, Martin M (2000) Success of traditional restocking of red-legged partridge for hunting purposes in areas of low density of northeast Spain Aragón. Z Jagdwiss 46:23–30

Grafen A (1990) Biologiacl signals as handicaps. J Theor Biol 144:517–546

Harashima K, Ohno T, Sawachika T, Hidaka T, Ohnishi E (1972) Carotenoids in orange pupae of the swallowtail, P*apilio xuthus*. Insect Biochem 2:29–48

Harashima K, Nakahara J, Kato G (1976) A new ketocarotenoid in integuments of orange pupae of a swallowtail, *Papilio xucthus*, and carapaces of a crab, *Paralithodes brevipes* (Hanasakigani in Japanese). Agric Biol Chem 40:711–717

Hart NS, Vorobyev M (2005) Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. J Comp Physiol A 191:381–392

Hill GE, Johnson JD (2012) The vitamin A–redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. Am Nat 180:E127–E150

LaFountain AM, Frank HA, Prum RO (2013) Carotenoids from crimson and maroon plumages of Old World orioles (Oriolidae). Arch Biochem Biophys 539:126–132

Lemoine Y, Schoefs B (2010) Secondary ketocarotenoid astaxanthin biosynthesis in algae: a multifunctional response to stress. Photosynth Res 106:155–177

Lozano GA (1994) Carotenoids, parasites, and sexual selection. Oikos 70:309–311

Mangoni O, Imperatore C, Tomas CR, Costantino V, Saggiomo V, Mangoni A (2011) The new carotenoid pigment moraxanthin is associated with toxic microalgae. Mar Drugs 9:242–255

Maoka T (2009) Recent progress in structural studies of carotenoids in animals and plants. Arch Biochem Biophys 483:191–195

Maoka T (2011) Carotenoids in marine animals. Mar Drugs 9:278–293

Matsuno T, Maoka T (1988) The carotenoids of crab *Paralithodes brevipes* (Hanasakigani in Japanese). Nippon Suisan Gakkaishi 54: 1437–1442

Matsuno T, Ookubo M (1982) The first isolation and identification of fritschiellaxanthin from a crab *Sesarma haematocheir* (AKATEGANI in Japanese). B Jpn Soc Sci Fish 48:653–659

McGraw KJ (2006) Mechanics of carotenoid-based coloration. In: Hill GE, McGraw KJ (eds) Bird coloration, vol 1, Mechanisms and measurements. Harvard University Press, Cambridge, pp 177–242

McGraw KJ, Gregory AJ (2004) Carotenoid pigments in male American goldfinches: what is the optimal biochemical strategy for becoming colourful? Biol J Linn Soc 83:273–280

McGraw KJ, Hardy LSJ (2006) Astaxanthin is responsible for the pink plumage flush in Franklin's and Ring-billed gulls. Field Ornithol 77: 29–33

Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans CA (1996) Antioxidant activities of carotenes and xanthophylls. FEBS Lett 384:240–242

Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: indicators of foraging efficiency, inmmunocompetence or detoxification ability? Avian Poult Biol Rev 11:137–159

Murillo E, McLean R, Britton G, Agócs A, Nagy V, Deli J (2011) Sapotexanthin, an A-provitamin carotenoid from Red Mamey (*Pouteria sapota*). J Nat Prod 74:283–385

Pérez-Bonilla A, Frikha M, Mirzaie S, García J, Mateos GG (2011) Effects of the main cereal and type of fat of the diet on productive performance and egg quality of brown-egg laying hens from 22 to 54 weeks of age. Poult Sci 90:2801–2810

Pérez-Rodriguez L (2009) Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays 31:1116–1126

Pérez-Rodriguez L, Viñuela J (2008) Carotenoid-based bill and eye ring coloration as honest signals of condition: an experimental test in the red-legged-partridge (*Alectoris rufa*). Naturwissenschaften 95:821–830

Rodríguez-Estival J, Martínez-Haro M, Martín-Hernando MP, Mateo R (2010) Sub-chronic effects of nitrate in drinking water on red-legged partridge (*Alectoris rufa*): Oxidative stress and T-cell mediated immune function. Environ Res 110:169–175

Stahl W, Sies H (2003) Antioxidant activity of carotenoids. Mol Asp Med 24:345–351

Stradi R (1998) The color of flight: carotenoids in bird plumage. Gruppo Editoriale Informatico Solei, Milan

Stradi R, Pini E, Celentano G (2001) Carotenoids in bird plumage: the complement of red pigments in the plumage of wild and captive bullfinch (*Pyrrhula pyrrhula*). Comp Biochem Physiol B 128:529–535

Suhnel S, Lagreze F, Ferreira JF, Campestrini LH, Maraschin M (2009) Carotenoid extraction from the gonad of the scallop *Nodipecten nodosus* (Linnaeus, 1758) (Bivalvia: Pectinidae). Braz J Biol 69: 209–215

Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham

Zahavi A (1975) Mate selection — a selection for a handicap. J Theor Biol 53:205–214