

Birds use fruit colour as honest signal of dietary antioxidant rewards

1 H. Martin Schaefer^{*,1}, K. McGraw² and C. Catoni¹

¹ Department of Evolutionary Biology and Animal Ecology, Faculty of Biology, University of Freiburg, Hauptstr. 1, 79104 Freiburg, Germany; and ² School of Life Sciences, Arizona State University, Tempe, AZ 85287 4501, USA

Summary

1. Fruits are among the most antioxidant-rich foods in nature and thus can be important dietary sources for combating oxidative stress in animals and humans.

2. Because fruits are pigmented by important plant antioxidants such as anthocyanins and carotenoids, frugivores may be able to gauge the antioxidant value of a fruit by its colour. However, although the importance of dietary antioxidants and oxidative stress is increasingly being acknowledged in evolutionary ecology, it is unknown whether animals can use visual signals to detect the presence of antioxidants in their food.

3. We studied the colour and pigment content of 60 bird-dispersed fruits and used an avian eye model to assess the ability of birds to visually discriminate carotenoid and anthocyanin contents of fruits. We then tested whether the frugivorous European blackcap (*Sylvia atricapilla*) preferentially consumes food containing anthocyanins.

4. Fruit colour variation was explained by variation in anthocyanin contents, and birds were capable of discriminating anthocyanin concentrations in fruits based on colour because fruits rich in anthocyanins are black or UV reflecting. However, we found that birds could not use colouration to determine the carotenoid contents of fruits. Ripe fruits contained more anthocyanins than unripe fruits, while there was no difference between ripe and unripe fruits in carotenoid contents. Anthocyanin contents correlated with caloric value in fruits, while there was no such correlation between carotenoids and total energy contents.

5. In food choice experiments, blackcaps selected food containing anthocyanins over food without anthocyanins.

6. In sum, our results indicate that birds actively selected for anthocyanins in their food and that they may use fruit colour as a foraging signal of anthocyanin antioxidant rewards.

Key-words: anthocyanins, carotenoids, plant–animal communication, oxidative stress, avian vision

Introduction

Plants depend on mobile animals as dispersal agents, and many plants offer nutritious fleshy fruits to attract dispersers to their reproductive organs. Fruit colours are traditionally viewed as an adaptation to increase the detectability of fruits for animals that disperse their seeds (Kerner 1895; Schmidt, Schaefer & Winkler 2004). This assumption seems intuitively plausible, given that fruits dispersed by diurnal animals typically change colour during ripening and become more conspicuous when ripe, at least to the human eye. Increasing

conspicuousness might not be the only strategy that plants use to attract seed dispersers to their fruits. At least in some communities, where plants compete for dispersal services by animals, plants use colour to signal the presence of macronutritional rewards (e.g. proteins, carbohydrates; Schaefer & Schmidt 2004). However, it is unknown whether plants generally use signals that indicate specific nutritional rewards to attract seed dispersers to their reproductive organs.

The most important pigments that impart colour in ripe fruits are chlorophyll, carotenoids and anthocyanins (Lancaster *et al.* 1997). Carotenoids and anthocyanins are important plant antioxidants that scavenge highly reactive oxygen species (ROS) (Gould, McKelvie & Markham 2002; Howitt & Pogson 2006). ROS are prone to react with other molecules causing

*Correspondence author.

E-mail: martin.schaefer@biologie.uni-freiburg.de

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1 oxidative stress by damaging DNA, proteins and lipids and
 2 thereby contributing to senescence and various degenerative
 3 diseases in plants and animals (Ames, Shigenaga & Hagen
 4 1993; Kong *et al.* 2003). Importantly, anthocyanins and
 5 carotenoids retain their antioxidant capacity when ingested
 6 by fruit-consuming animals. These dietary antioxidants are
 7 therefore key elements to combat oxidative stress, which
 8 animals face in every step of their life because ROS are con-
 9 tinuously produced by the body, particularly during periods
 10 of high metabolic rate or detoxification (von Schantz *et al.*
 11 1999). Dietary antioxidants, such as anthocyanins and caro-
 12 tenoids, might therefore constitute an important nutritional
 13 reward for fruit-eating animals. Moreover, because these pig-
 14 ments both impart colour and serve as antioxidants, plants
 15 might increase their chances of successful seed dispersal by
 16 attracting mutualistic seed dispersers with honest signals of
 17 antioxidant contents (Schaefer, Schaefer & Levey 2004). The
 18 lone study on this subject was conducted on a limited number
 19 of grocery-sold fruits, but found that humans might discern
 20 anthocyanin and chlorophyll, but not carotenoid, concentra-
 21 tions using colour (Lancaster *et al.* 1997).

22 Several studies have proposed that colourful animals like
 23 birds and fish use the colour of foods as a signal of carotenoid
 24 contents and thus as a means of acquiring pigments for
 25 developing bright coloration or for improving immune func-
 26 tions (reviewed in: McGraw 2006). While animals do not use
 27 anthocyanins for pigmentation, these might be more impor-
 28 tant contributors to immune functions owing to their higher
 29 antioxidant capacity compared to carotenoids approximately
 30 [c. 4.4 vs. 0.2–2.9 Trolox equivalent antioxidant capacity
 31 (TEAC); Miller *et al.* 1996; Rice-Evans, Miller & Paganga
 32 1996]. However, no experiments to date have addressed
 33 whether or not fruit-eating animals select food to increase
 34 anthocyanin intake.

35 We had two overall aims that motivated our study: (i) to test
 36 the hypothesis that fruit colour honestly signals antioxidant
 37 contents, and (ii) to test the hypothesis that animals use
 38 colour signals to increase antioxidant intake. To test the first
 39 hypothesis, we combined biochemical, spectrophotometric
 40 and vision-modelling methods to study the relationship between
 41 colour and the contents of carotenoids and anthocyanins in
 42 bird-dispersed fruits. Given that chlorophyll might mask the
 43 presence of pigments with antioxidant function, we tested
 44 whether interspecific variation in fruit reflectance is explained
 45 by differences in the contents of carotenoids and anthocyanins.
 46 We then used an avian vision model (Vorobyev & Osorio
 47 1998) to investigate whether birds, the most ubiquitous group
 48 of frugivores, can use fruit colour variation to discriminate
 49 fruits according to their pigment contents. To account for
 50 other potential signalling functions of fruit pigments, we also
 51 compared the carotenoid and anthocyanin contents of ripe
 52 and unripe fruits, analysed the relationship between pigments
 53 and fruits' caloric value and analysed whether the concentra-
 54 tion of pigments influenced the contrasts between fruits and
 55 their background.

56 To test our second aim, that birds use fruit colour signals
 57 to increase their intake of antioxidants, we experimentally

examined food choice in blackcaps (*Sylvia atricapilla*), a
 common seed disperser for many European plants (Jordano
 1987). Specifically, we tested whether these birds preferen-
 tially consume food with anthocyanins.

Materials and methods

FRUIT COLLECTION

We determined pigment composition in ripe fruits from 60 plant
 species originating from 18 families (Table 1). In 10 plant species, we
 also analysed pigment composition in unripe fruits. We used a com-
 parative interspecific approach because birds commonly consume
 a variety of ripe fruits (e.g. more than 100 fruit species in salvin's
 curassow (*Mitu salvini*) (Santamaria & Franco 2000), whereas
 unripe fruits are rarely consumed (Schaefer & Schaefer 2006). From
 August 2003 to October 2004, fruits ($n = 30$ species) were collected
 in Germany and in Mediterranean regions. Additionally we collected
 fruits of 30 species in the Botanical Gardens of the University of
 Freiburg. These species originated from temperate areas, mainly
 Asia, and belonged to the same families as our sample of fruits
 native to Europe. None of the plants collected in the Botanical
 Gardens were bred as ornamentals. We included these fruits to
 derive general conclusions on fruit colour and pigment composition
 that hold for a larger diversity of fruits.

To determine the relationship between fruit pigments and the
 caloric content of fruits, we used 27 of the fruit species described
 above, for which we had additional information on the nutritional
 composition of fruit pulp from the fruit data base assembled by
 Pedro Jordano (2001). To increase sample size for this analysis, we
 used a further 33 species of ripe fruits with known caloric value and
 pigment composition from Venezuela (Schaefer *et al.* 2003), total-
 ling 60 species. None of the fruit species included in the analyses is
 of commercial interest, and all species are dispersed by birds (see
 Schaefer *et al.* 2003; Schaefer, Schaefer & Vorobyev 2007). We did
 not address phylogenetic constraints on colour and fruit com-
 pounds in this study because in previous studies on fruit colour and
 nutritional compounds we failed to find a significant phylogenetic
 signal in our sample (Schaefer *et al.* 2003; Schaefer *et al.* 2007).

COLOUR MEASUREMENTS

We measured the reflectance spectra of 20 ripe fruits from each
 species with an Ocean Optics (Dunedin, FL) USB2000 spectrometer
 and a Top Sensor System Deuterium-Halogen DH-2000 as a stand-
 arized light source. For each species, we also measured the reflect-
 2
 ance of 10 background structures against which fruits are displayed
 (e.g. leaves and bark of each species). Reflectance was measured
 relative to a standard white reference tile (Top Sensor Systems WS-
 2). For colour measurements, we used a coaxial fiber cable (QR400-
 7, Ocean Optics) that was mounted inside a matt black plastic tube
 to exclude ambient light (Schaefer *et al.* 2007). The angle of illu-
 mination and reflection was fixed at 45° to minimize glare. Spectra
 were processed with SPECTRA WIN 6.0 software and calculated in 5-
 nm intervals from 300–700 nm. 3

AVIAN EYE MODEL

To assess fruit colour according to avian vision, we analysed fruit
 reflectance spectra using an eye model that is based on the spectral
 sensitivities and the receptor noise of the four cone types that are

1 **Table 1.** List of the anthocyanin and carotenoid contents (mg g⁻¹) of
2 all fruits species

3	4 Genus	5 Species	Anthocyanin contents	Carotenoid contents
6	<i>Amelanchier</i>	<i>lamarkii</i>	11.66	0.02
7	<i>Aronia</i>	<i>prunifolia</i>	7.68	0.35
8	<i>Berberis</i>	<i>thunbergii</i>	11.73	0.36
9	<i>Bryonia</i>	<i>dioica+</i>	1.69	1.89
10	<i>Clerodendrum</i>	<i>trichotomum</i>	6.30	0.01
11	<i>Cornus</i>	<i>alba</i>	0.11	0.19
12	<i>Cornus</i>	<i>ammomum</i>	2.38	0.19
13	<i>Cornus</i>	<i>kousa</i>	0.15	0.42
14	<i>Cornus</i>	<i>mas+</i>	4.10	0.12
15	<i>Cornus</i>	<i>racemosa+</i>	0.25	0.09
16	<i>Cornus</i>	<i>sanguinea+</i>	35.29	0.98
17	<i>Cotoneaster</i>	<i>affinis</i>	0.85	0.24
18	<i>Cotoneaster</i>	<i>dammeri</i>	1.62	0.24
19	<i>Cotoneaster</i>	<i>dielsianus</i>	0.43	0.42
20	<i>Cotoneaster</i>	<i>melanocarpa</i>	28.78	0.42
21	<i>Cotoneaster</i>	<i>mouppensis</i>	21.06	0.45
22	<i>Cotoneaster</i>	<i>nebrodensis</i>	0.60	0.55
23	<i>Cotoneaster</i>	<i>sp.</i>	0.66	0.19
24	<i>Crataegomespilus</i>	<i>dardani</i>	0.56	0.16
25	<i>Crataegomespilus</i>	<i>potsdamii</i>	0.48	0.25
26	<i>Crataegus</i>	<i>laevigata+</i>	0.60	0.26
27	<i>Crataegus</i>	<i>sp.</i>	38.31	0.21
28	<i>Crataegus</i>	<i>sp.</i>	0.08	0.06
29	<i>Elaeagnus</i>	<i>multiflora</i>	0.05	0.95
30	<i>Hedera</i>	<i>helix+</i>	1.22	1.14
31	<i>Hippophae</i>	<i>rhamnoides</i>	0.07	0.57
32	<i>Idesia</i>	<i>polycarpa</i>	1.22	0.46
33	<i>Ligustrum</i>	<i>vulgare+</i>	2.67	0.41
34	<i>Lonicera</i>	<i>alpigena</i>	0.23	0.10
35	<i>Lonicera</i>	<i>nigra</i>	3.35	0.50
36	<i>Lonicera</i>	<i>ruprechtiana</i>	0.19	0.73
37	<i>Lonicera</i>	<i>xylosteum+</i>	1.40	0.30
38	<i>Morus</i>	<i>nigra+</i>	37.65	0.04
39	<i>Parthenocissus</i>	<i>quinquefolia+</i>	16.46	0.42
40	<i>Phillyrea</i>	<i>angustifolia+</i>	2.67	0.05
41	<i>Phytolacca</i>	<i>americana+</i>	17.94	0.43
42	<i>Polygonatum</i>	<i>multiflorum+</i>	0.58	2.93
43	<i>Prunus</i>	<i>avium+</i>	19.56	0.04
44	<i>Prunus</i>	<i>domestica+</i>	6.20	0.17
45	<i>Prunus</i>	<i>lauocerasus+</i>	25.36	0.10
46	<i>Prunus</i>	<i>mahaleb+</i>	15.83	0.27
47	<i>Prunus</i>	<i>sp.</i>	1.10	0.41
48	<i>Prunus</i>	<i>yendoensis</i>	22.21	0.03
49	<i>Pyracantha</i>	<i>coccinea+</i>	0.30	0.68
50	<i>Rhamnus</i>	<i>japonicus</i>	6.27	0.97
51	<i>Rosa</i>	<i>corymbifera</i>	0.94	1.09
52	<i>Rosa</i>	<i>glauca+</i>	0.46	2.22
53	<i>Rosa</i>	<i>pimpinellifolia</i>	39.21	0.05
54	<i>Rosa</i>	<i>rubigenosa</i>	1.14	1.08
55	<i>Rosa</i>	<i>vil+</i>	0.08	1.23
56	<i>Sambucus</i>	<i>canadensis+</i>	39.61	0.35
57	<i>Sambucus</i>	<i>racemosa+</i>	5.53	0.25
58	<i>Sorbus</i>	<i>aria+</i>	0.50	0.38
59	<i>Sorbus</i>	<i>aucuparia+</i>	1.02	1.26
60	<i>Streptopus</i>	<i>amplexifolius</i>	3.29	3.05
61	<i>Tamus</i>	<i>communis+</i>	1.29	2.71
62	<i>Taxus</i>	<i>baccata</i>	1.38	0.41
63	<i>Viburnum</i>	<i>lantana+</i>	1.85	0.19
64	<i>Viburnum</i>	<i>carlesii</i>	2.41	0.18
65	<i>Viburnum</i>	<i>opulus+</i>	2.92	1.36

66 The symbol '+' indicates those species that were used for assessing the
67 relationship between pigment contents and energy contents in fruits.

assumed to function in avian colour discrimination (Vorobyev & Osorio 1998). Based on analytical approximation of cone visual pigments and oil droplet spectra, the model calculates cone excitation values for each fruit colour spectrum. The chromatic contrasts between fruit and background were calculated as the log of the quotient of quantum catches of photoreceptors from both spectra [see Vorobyev *et al.* (1998) for equations]. The chromatic contrasts describe how much two spectra are separated in receptor space. The units for chromatic contrasts are just noticeable differences (jnds). One jnd is at the threshold of discrimination, while values less than one jnd indicate that two colours are indistinguishable, and as values increase above one jnd, objects become easier to discriminate. Our model is based on the spectral sensitivities of the blue tit (*Cyanistes caeruleus*) with a UVS cone (Hart *et al.* 2000), but the results are also representative for birds with different short-wave visual sensitivities (VS cone; data not shown).

PIGMENT ANALYSIS

Seeds and fruit pulp were separated, and fruit pulp was frozen immediately afterwards for all 60 species. The additional 33 species from Venezuela were dried prior to pigment analysis. The dual anthocyanin/carotenoid extraction procedure was adapted from Lazcano, Yoo & Pike (2001). We weighed out c. 1 g of fresh frozen or dried fruit material with an electronic balance to the nearest 0.0001 g and added the fruit to a zirconia jar equipped with zirconia grinding balls. We then added 2 mL of 97.5% ethanol in 6 N HCl and ground the fruit for 3 min at 30 Hz in a Retsch MM200 mixer mill (Haan, Germany). After grinding, we transferred the extract to a fresh 9-mL screw-cap tube and rinsed the jar with 1 mL acidified ethanol to recover any residual extract. We then added 2 mL hexane, shook the tube vigorously for 1 min, and centrifuged the tube at 1500 r.p.m. for 3 min. After centrifuging, we transferred the upper carotenoid-containing supernatant (hexane) to a clean culture tube and transferred the upper precipitate, along with the lower anthocyanin-containing supernatant (acidified ethanol), to a 3-mL syringe fitted with a 0.45-micron nylon filter. We filtered the anthocyanin extract into a clean culture tube, and then evaporated the solvent in both culture tubes to dryness under a stream of nitrogen. We resuspended the pigments in 1 mL acidified ethanol (for anthocyanins) or 1 mL hexane (for carotenoids) and measured light absorbance of the solutions in quartz cuvettes at the lambda-max for each sample with a Beckman-Coulter DU 520 UV/Vis spectrophotometer (Fullerton, CA). Concentrations are reported as milligrams of pigment per gram of fruit. Assays were run in duplicate for all fruits, and results were highly repeatable for both types of pigments (bivariate correlation analysis: anthocyanins: $r = 0.98$, $P < 0.001$; carotenoids: $r = 0.95$, $P < 0.001$). We used mean values in statistical analyses.

FOOD CHOICE EXPERIMENT

To test whether birds select food based on anthocyanin contents, we conducted a food choice experiment with 11 captive female black-caps on four consecutive days in May 2007. The birds were 1 year old, hand-raised and maintained in individual standard cages with a 14 : 10 h light/dark cycle at the Institute of Avian Research in Wilhelmshaven, Germany. Birds were kept during that period on a beige semisynthetic standard food without anthocyanins (Bairlein 1986). During the experiment, we supplemented anthocyanins using an extract (Antho50, Ferlux S.A., Cournon d'Auvergne, France) containing at least 50% anthocyanins, other polyphenols (16%),

1 organic acids (12.5%), water (4.7%), sugar (4.5%), proteins (0.9%)
 2 and other trace substances. Food with the extract had no specific
 3 smell or taste for humans. The sugar added with the extract amounted
 4 to only 0.002% of the total food weight, and we assume that such
 5 difference did not influence avian food choice.

6 To test food choice, we provided two 5-cm-deep green cups, so
 7 that the food was exclusively seen against the green background that
 8 simulated the colour of background vegetation. We measured the
 9 reflectance spectra of both foods and food cups as described above
 10 and calculated the chromatic contrasts between both. Cups contained
 11 17.6 ± 0.32 g (mean \pm SE) of standard food each. One of the foods
 12 was bluish containing 5 mg of anthocyanins (0.28 mg g^{-1} fresh food),
 13 while the other one was void of anthocyanins and beige in colour.
 14 The concentration of anthocyanins is likely biologically relevant as
 15 it corresponds to low anthocyanin concentrations in fruits. Each
 16 day, we weighed the cups after 1 h to assess preference and again
 17 after 24 h, when there is more opportunity for metabolic feedbacks.
 18 We exchanged food cups every 24 h. Since the results were consistent
 19 over both time scales, we report the values obtained after 24 h. In all
 20 measurements, we corrected for evaporation using two control
 21 food cups.

22 STATISTICAL ANALYSES

23 We tested the hypothesis that plants signal the contents of anti-
 24 oxidants based on the reflectance spectra of fruit colours. As a first
 25 step, we reduced the many correlated variables of fruit reflectance
 26 (80 variables of mean reflectance per 5-nm intervals) by conducting
 27 a principal component analysis (PCA), which transforms the corre-
 28 lated variables into few orthogonal variables [principal components
 29 (PC)]. Each PC is a weighted linear sum of the original data set,
 30 being therefore a mathematical transformation of the original data
 31 rather than a statistical summary (Cuthill *et al.* 1999). The PC coefficients
 32 are the weights that relate the original variables (5-nm intervals)
 33 to the PCs. Moreover, PCs are by definition independent from
 34 each other, facilitating further analyses. Applying PCA to reflectance
 35 data normally yields one to three PCs that capture 90%–99% of
 36 variance (Endler 1990). Thus, PCA greatly reduces the number of
 37 variables of reflectance spectra while keeping the loss of information
 38 to a minimum.

39 To determine the relationship between fruit colour and pigment
 40 composition, we entered the first three PCs as independent variables
 41 into multiple regressions with anthocyanin and carotenoid concen-
 42 trations as dependent variables. Multiple regressions are suitable
 43 because all explanatory variables were included in the analysis.
 44 Regressions were performed separately for anthocyanins and caro-
 45 tenoids as stepwise backwards and forward procedures, which
 46 yielded identical results.

47 To study whether birds perceive fruits differently depending on
 48 their pigment composition, we entered ln-transformed cone excita-
 49 tion values as independent variables in stepwise backwards (and
 50 forward) multiple regressions, with anthocyanin or carotenoid con-
 51 tents as dependent variables. To compare birds' food choice of
 52 alternative foods and the pigment composition of ripe and unripe
 53 fruits of the same species, we used paired *t*-tests. To compare whether
 54 fruits originating from the Botanical Gardens differ from fruits
 55 collected in Europe, we used a nonparametric Mann–Whitney test.
 56 Moreover, to assess whether birds might use pigment concentration
 57 as a signal of other aspects of fruit nutritional quality, we used a
 bivariate correlation analysis to determine whether the contents of
 anthocyanins and carotenoids correlated with each other or with the
 caloric value of fruits. Finally, we tested whether the contents of

anthocyanins and carotenoids influenced the contrasts between
 fruits and their background (leaves and bark) using a multiple linear
 regression, with fruit contrasts as dependent variable and the con-
 tents of anthocyanins and carotenoids as independent variables.

Results

FRUIT COLOUR AND PIGMENT COMPOSITION

PCA yielded three components that explained 97% of total
 variance in fruit colour. PC1 explained 70% of variance in
 fruit colours. The coefficients relating PC1 to the original
 data were all positive and of similar magnitude; PC1 thus
 reflected achromatic variation in fruit reflectance (Fig. 1). PC2
 accounted for 21% of total variance, with negative coefficients
 below 540 nm and positive coefficients at longer wavelengths.
 PC2 thus captured variation in the relative amount of short-
 to long-wavelength reflectance, so that fruits with high UV
 reflectance or black fruits with low overall change in reflectance
 had negative PC2 values and fruits with a low relative
 reflectance at short-wavelengths (white without UV reflectance
 and intensively coloured red and orange fruits) had high
 PC2 values. PC3 explained 6% of total variance and was char-
 acterised by positive coefficients from 300–430 nm and above
 615 nm and negative coefficients from 435–610 nm. It repre-
 sented thus the amount of variation in mid-wavelengths rela-
 tive to reflectance at shorter and longer wavelengths.

Fruits collected in Europe and the Botanical Gardens did
 not differ in the contents of carotenoids or anthocyanins
 (Mann–Whitney test, $z = -0.48$ and $z = -1.62$, both $P > 0.05$).
 Fruits contained $0.57 \pm 0.08 \text{ mg g}^{-1}$ (mean \pm SE; range 0.012–
 3.04) carotenoids and on average 14 times more anthocyanins
 ($7.53 \pm 1.4 \text{ mg g}^{-1}$; range 0.05–39.61). In stepwise multiple
 regressions, PCs of fruit colour variance significantly predicted

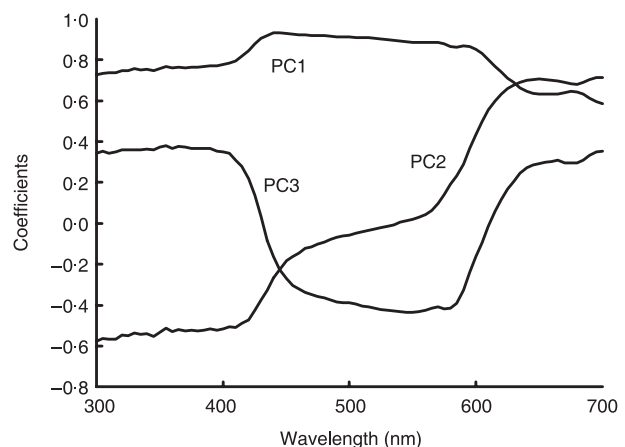


Fig. 1. Principal component (PC) coefficients relating PCs to the original reflectance spectra of 60 fruit species. High positive values at a given wavelength indicate a strong association between the specific PC and that wavelength (e.g. across the entire range for PC1). Likewise, negative values of -0.6 in PC2 indicate a negative association between reflectance in the UV (range 300–400 nm) and PC2. Values around 0 indicate that there is no association between a PC and a given wavelength.

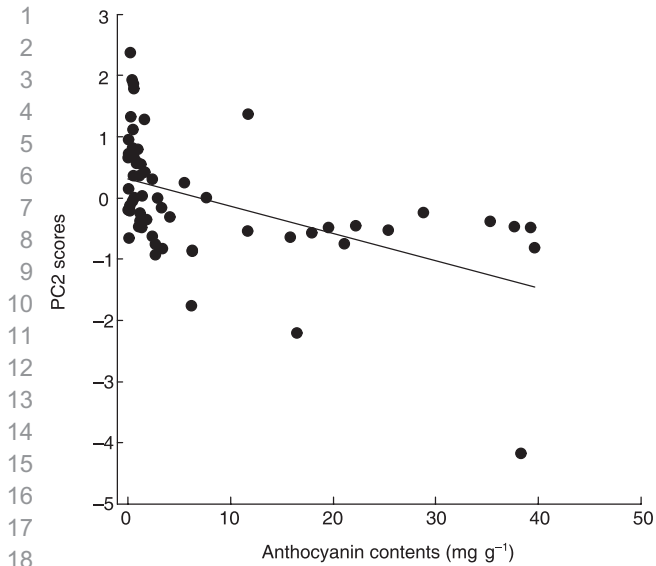


Fig. 2. There is a negative relationship between anthocyanin contents in fruits and the relative amount of short- to long-wavelength reflectance (PC2). Black fruits and fruits reflecting UV have negative PC2 scores, whereas red and orange fruits have positive PC2 scores. Note the extensive variation in PC2 scores, particularly in fruits with low anthocyanin contents.

variation in anthocyanin contents (final model: $N = 60$, $r^2 = 0.25$; $F = 20.21$, $P < 0.0001$). There was no correlation between PC1 or PC3 and anthocyanin contents, but PC2 correlated negatively with anthocyanin contents ($t = -4.02$, $P < 0.001$; Fig. 2), indicating that fruits with high UV reflectance or black fruits had high contents of anthocyanins, whereas red/orange and white fruits without UV reflectance had low contents of anthocyanins. Multiple regressions on PCs and carotenoid contents yielded no significant relationships (all PCs excluded from analysis in backwards procedure or none included in forward analysis).

FRUIT PIGMENTS AND RIPENESS

Ripe fruits contained more anthocyanins ($18.57 \pm 5.23 \text{ mg g}^{-1}$) than unripe fruits ($5.98 \pm 1.37 \text{ mg g}^{-1}$) of the same species (paired t -test, $N = 10$, $t = 2.69$, $P < 0.05$). However, ripe and unripe fruits did not differ in their contents of carotenoids (1.14 ± 0.53 vs. $1.52 \pm 0.66 \text{ mg g}^{-1}$, respectively; paired t -test, $N = 10$, $t = -0.57$, $P > 0.4$). The contents of anthocyanins correlated with total energy contents in fruits ($N = 60$, $r = 0.363$, $P < 0.01$), whereas there was no correlation between anthocyanins and carotenoids or carotenoids and total energy contents ($N = 60$, $r = 0.08$ and $r = 0.18$, both $P > 0.16$).

AVIAN VISION AND PIGMENT COMPOSITION

Variation in anthocyanin contents is captured by cone output values of the avian eye (multiple regressions, final model: $N = 60$, $r^2 = 0.27$; $F = 10.88$, $P < 0.0001$). Only the output of the long-wavelength cone corresponded to anthocyanin contents ($t = -4.54$, $P < 0.001$; Fig. 3), while there were no effects

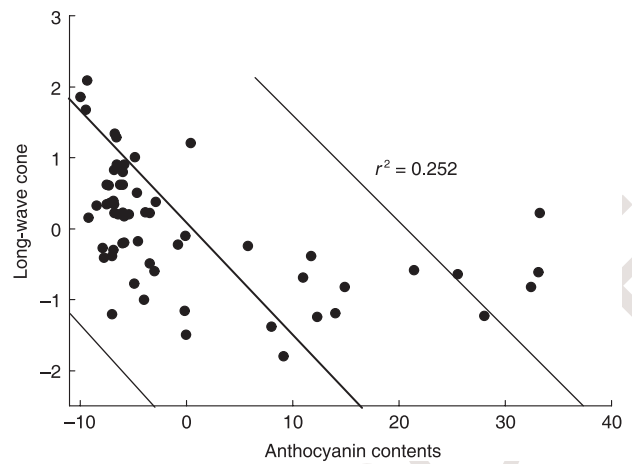


Fig. 3. Partial regression plot for the relationship between anthocyanin contents and long-wave cone outputs, the linear regression is shown with 95% CIs.

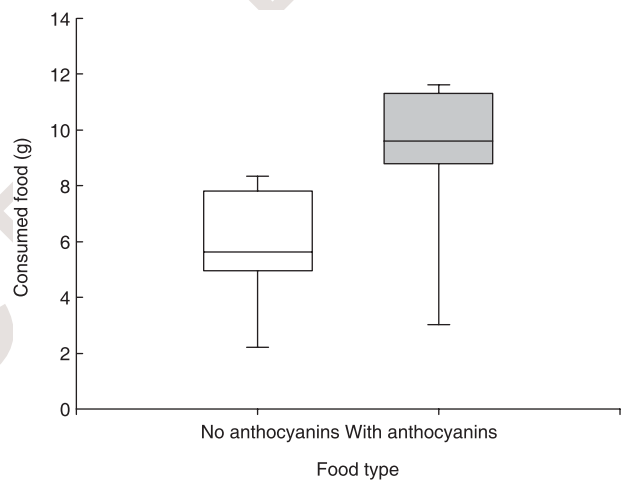


Fig. 4. Birds selected food with anthocyanins over food of identical nutrient composition without anthocyanins. Birds consumed on average 9.6 g (9.1–11.1, 2nd and 3rd quartile) of food with anthocyanins and 5.6 g (5.0–6.7) of control food per day. Illustrated is the mean food intake during the four consecutive days of the experiment.

for the UV- and the middle-wavelength cones (both excluded from final model) and a marginal effect for the short-wavelength cone ($t = 1.88$, $P = 0.062$). Variation in carotenoid contents is not reliably indicated by cone output values of the avian eye (multiple regressions, final model: $N = 60$, $r^2 = 0.12$; $F = 2.62$, $P = 0.06$). There was also no relationship between the contents of anthocyanins and carotenoids and the contrasts between fruit and background (multiple regressions: $r^2 = 0.06$; $F = 1.82$, $P = 0.17$).

FOOD CHOICE EXPERIMENT

Birds selected food containing anthocyanins over food of identical nutritional composition without anthocyanins (t -test, $N = 11$, $t = 4.91$, $P = 0.001$; Fig. 4). Food with anthocyanins

1 was less contrasting (6.9 jnds) against the cups than food
2 without anthocyanins (15.2 jnds).

4 Discussion

6 Our biochemical and spectrophotometric analyses showed
7 that the colour of bird-dispersed fruits correlated with the
8 contents of anthocyanins but not with carotenoid concen-
9 trations. Modelling the visual abilities of birds similarly
10 demonstrated that birds are able to discriminate visually
11 between fruits of different anthocyanin contents but that they
12 could not discriminate fruits according to their carotenoid
13 contents. Finally, we demonstrated that frugivorous birds
14 actively select food containing anthocyanins.

17 FRUIT COLOUR AS HONEST SIGNAL OF 18 ANTHOCYANINS

19 Although our study shows that anthocyanin concentrations
20 increase during fruit ripening, we found no relationship
21 between anthocyanin contents and the contrasts between
22 fruits and their background. This result challenges the pre-
23 vailing notion that the increase in fruit pigmentation during
24 ripening is an adaptation to facilitate detection by diurnal
25 seed dispersers (e.g. Kerner 1895; Schmidt *et al.* 2004). The
26 result that increasing anthocyanin concentrations do not con-
27 comitantly increase conspicuousness appears counterintui-
28 tive, but it is supported by an experiment documenting that
29 some birds can detect red fruits that have typically low con-
30 tents of anthocyanins from a larger distance than black fruits
31 that are characterised by high contents of anthocyanins
32 (Schaefer *et al.* 2006).

33 Anthocyanins are common skin colorants in fruits, and
34 thus it is not surprising that fruit colour correlated with
35 anthocyanin concentrations. Anthocyanin concentrations in
36 grocery-store fruits are also predicted by a measure of colour
37 (hue; Lancaster *et al.* 1997). Birds reliably distinguish the
38 anthocyanin contents of fruits based upon the excitation
39 values of the avian long-wave receptor. Low relative excitation
40 values correspond to blue, black and UV-reflecting fruits,
41 which are rich in anthocyanins and have low reflectance in the
42 spectral area of the long-wave receptor.

43 Owing to the consistent correlation between colour and
44 anthocyanin contents, we suggest that blackcaps used the col-
45 our differences between the foods as a proximate cue to select
46 anthocyanins in our food choice experiment. Food selection
47 for anthocyanins is not explicable by innate colour preference
48 because blackcaps innately prefer red, not blue food items
49 (Schmidt & Schaefer 2004). Since the food containing
50 anthocyanins was less contrasting than the alternative food,
51 we can exclude that selection for anthocyanins is biased by
52 preferences to consume more conspicuous food (see Schmidt
53 *et al.* 2004). At the evolutionary level, birds might have
54 selected anthocyanins because they indicate high caloric
55 rewards, as shown by the correlation between anthocyanin
56 concentrations and energy contents in fruits. However, we
57 consider this explanation unlikely to explain our results

because both foods had the same caloric content during four
consecutive days. In such a time span, birds quickly adjust
their food choice according to the energy contents of alterna-
tive foods (Schaefer *et al.* 2003).

We thus hypothesise that, ultimately, birds choose antho-
cyanins to increase the intake of dietary antioxidants and thus
to improve health. This conjecture is supported by the fact
that anthocyanins are health stimulants in humans and mice
(Kong *et al.* 2003) and by a recent experiment documenting
that anthocyanins improve immune functions in frugivorous
birds (Catoni *et al.* unpublished data). Fruits are generally a
rich source of different antioxidants, which is the main reason
why their consumption and that of red wine is considered
beneficial for human health (Heinonen, Meyer & Frankel
1998). Importantly, anthocyanins are among the most potent
groups of dietary antioxidants (Kong *et al.* 2003), and they
are the most visible sign of antioxidant capacity in fruits
because their presence is correlated to that of other colourless
antioxidants (Zheng & Wang 2003). As a consequence, birds
might also select anthocyanins because they indicate high
overall contents of colourless antioxidants. Regardless of the
exact mechanism, our food choice experiment is the first evi-
dence that birds choose food on the basis of anthocyanins.
Thus, we suggest a new hypothesis, which predicts that selec-
tion for anthocyanin contents is an important determinant of
fruit consumption.

If we consider the signalling potential of anthocyanins
from a plant's perspective, we suggest that plants can increase
their reproductive success because anthocyanins are simul-
taneously a visual signal and an indicator of antioxidant
activity (see Schaefer *et al.* 2004). In general, some plants
signal honestly nutritional rewards such as macronutrients or
energy (this study) to attract seed dispersers and pollinators
to their reproductive organs (Schaefer & Schmidt 2004;
Armbruster, Antonsen & Pélabon 2005). If, as in the case of
anthocyanins, the predominant pigments themselves consti-
tute a potential reward, the signal is honest because of the
unavoidable connection between signal intensity and quality.
Our hypothesis that birds preferentially consume fruits con-
taining anthocyanins is supported at least partly by previous
experiments. Adult redwings (*Turdus iliacus*) prefer UV-
reflecting fruits (which are associated with high anthocyanin
contents) over non-UV-reflecting fruits that are otherwise
identical (Siitari, Honkavaara & Viitala 1999), and American
robins (*Turdus migratorius*) – as well as humans (Crisosto,
Crisosto & Mertheney 2003) – exhibit strong preferences for
dark purple hues (corresponding to high anthocyanin con-
tents) that even overrule preferences for energy-rich fruits
(Willson 1994).

CAROTENOIDS AND THE PROXIMATE BASIS OF FRUIT COLOURS

Fruits are the resource with the highest contents of caroten-
oids (Olson & Owens 2005), but we show that fruit colour is
not a reliable signal of carotenoid contents for birds. Birds are
unlikely to be able to discriminate carotenoid contents in

1 interspecific comparisons of ripe fruits. This result is sup-
 2 ported by a previous study on grocery-sold fruits which found
 3 no relationship between carotenoids and colour (Lancaster
 4 *et al.* 1997). Moreover, carotenoid contents did not differ
 5 between ripe and unripe fruits in our study, further corrobo-
 6 rating that carotenoid contents are not directly linked to fruit
 7 colouration. Consequently, birds aiming to optimize carote-
 8 noid intake for coloration or health purposes must rely on
 9 tactics other than visual assessment of fruit colour. An influ-
 10 ential hypothesis suggests that sensory biases for orange and
 11 red nuptial colouration may have evolved in a foraging con-
 12 text to optimise carotenoid intake from fruits (Rodd *et al.*
 13 2002). Given the lack of a relationship between fruit colour
 14 and carotenoid contents, our results suggest that sensory
 15 biases are unlikely to evolve for that reason, at least in animals
 16 that consume a variety of differently coloured fruits.

17 One proximate explanation for the lack of a relationship
 18 between colour and carotenoid contents is the co-occurrence
 19 of anthocyanins and chlorophyll in fruit skin. Chlorophylls,
 20 which are the main pigments in the skin of unripe fruits, might
 21 mask carotenoids in some ripe fruits, as they do in leaves (see
 22 Ougham, Morris & Thomas 2005). For example, fruits with
 23 low contents of anthocyanins show a characteristic dip in
 24 reflectance at 670 nm (Fig. 2) that indicates the presence of
 25 chlorophyll (see also Sumner & Mollon 2000). Carotenoids
 26 might also be masked by anthocyanins. For instance, high
 27 concentrations of anthocyanins mask carotenoid contents in
 28 black fruits, regardless of their concentration. On average,
 29 anthocyanins were 14× more concentrated than carotenoids
 30 in the fruits we studied and they impart more colour per
 31 molecule than do carotenoids (based on their extinction coeffi-
 32 cients; Sims & Gamon 2002). Moreover, even red colouration
 33 is not a reliable indicator of carotenoid contents, as caroten-
 34 oids are nearly absent in many red fruits (e.g. strawberries;
 35 Paiva *et al.* 1998) and colours are produced by low concen-
 36 trations of anthocyanins. The proximate explanation for why
 37 anthocyanins are revealed by two different colours (blue-black
 38 and UV) is that in temperate fruits, UV reflectance is caused
 39 by epicuticular wax layers that cover the fruit (Burkhardt
 40 1982). These wax layers are usually associated with the blue or
 41 black skin colour that is produced by high concentrations of
 42 anthocyanin pigments *per se*.

43 Conclusions

44
 45
 46 In summary, we propose that birds can visually discriminate
 47 fruits on the basis of their antioxidant capacity. We docu-
 48 mented that birds actively select anthocyanins in their food.
 49 Consequently, plants might use anthocyanin pigmentation to
 50 signal antioxidant activity to seed dispersers and thereby
 51 increase their reproductive success. Future experiments are
 52 required to test whether higher antioxidant capacity in fruits
 53 increases their chances to be consumed by seed dispersers. So
 54 far, the health-stimulating effects of anthocyanins have been
 55 largely ignored in evolutionary ecology, although this has
 56 emerged as a prolific research field in medical and nutritional
 57 sciences.

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