

Birds use fruit colour as honest signal of dietary antioxidant rewards

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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 macro-nutritional 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

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

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

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

To test our second aim, that birds use fruit colour signals 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 preferentially 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 comparative 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, Schmidt & Winkler 2003a), totalling 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.* 2003a; Schaefer, Schaefer & Vorobyev 2007). We did not address phylogenetic constraints on colour and fruit compounds 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.* 2003a; 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 an Ocean Optics Deuterium Halogen DH-2000 as a standardized light source. For each species, we also measured the reflectance 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 illumination and reflection was fixed at 45° to minimize glare. Spectra were processed with SPECTRA WIN 4.1 software (Ocean Optics) and calculated in 5-nm intervals from 300–700 nm.

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

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

Genus	Species	Anthocyanin contents	Carotenoid contents
<i>Amelanchier</i>	<i>lamarkii</i>	11.66	0.02
<i>Aronia</i>	<i>prunifolia</i>	7.68	0.35
<i>Berberis</i>	<i>thunbergii</i>	11.73	0.36
<i>Bryonia</i>	<i>dioica+</i>	1.69	1.89
<i>Clerodendrum</i>	<i>trichotomum</i>	6.30	0.01
<i>Cornus</i>	<i>alba</i>	0.11	0.19
<i>Cornus</i>	<i>ammomum</i>	2.38	0.19
<i>Cornus</i>	<i>kousa</i>	0.15	0.42
<i>Cornus</i>	<i>mas+</i>	4.10	0.12
<i>Cornus</i>	<i>racemosa+</i>	0.25	0.09
<i>Cornus</i>	<i>sanguinea+</i>	35.29	0.98
<i>Cotoneaster</i>	<i>affinis</i>	0.85	0.24
<i>Cotoneaster</i>	<i>dammeri</i>	1.62	0.24
<i>Cotoneaster</i>	<i>dielsianus</i>	0.43	0.42
<i>Cotoneaster</i>	<i>melanocarpa</i>	28.78	0.42
<i>Cotoneaster</i>	<i>moupenis</i>	21.06	0.45
<i>Cotoneaster</i>	<i>nebrodensis</i>	0.60	0.55
<i>Cotoneaster</i>	<i>sp.</i>	0.66	0.19
<i>Crataegomespilus</i>	<i>dardani</i>	0.56	0.16
<i>Crataegomespilus</i>	<i>potsdamii</i>	0.48	0.25
<i>Crataegus</i>	<i>laevigata+</i>	0.60	0.26
<i>Crataegus</i>	<i>sp.</i>	38.31	0.21
<i>Crataegus</i>	<i>sp.</i>	0.08	0.06
<i>Elaeagnus</i>	<i>multiflora</i>	0.05	0.95
<i>Hedera</i>	<i>helix+</i>	1.22	1.14
<i>Hippophae</i>	<i>rhamnoides</i>	0.07	0.57
<i>Idesia</i>	<i>polycarpa</i>	1.22	0.46
<i>Ligustrum</i>	<i>vulgare+</i>	2.67	0.41
<i>Lonicera</i>	<i>alpigena</i>	0.23	0.10
<i>Lonicera</i>	<i>nigra</i>	3.35	0.50
<i>Lonicera</i>	<i>ruprechtiana</i>	0.19	0.73
<i>Lonicera</i>	<i>xylosteum+</i>	1.40	0.30
<i>Morus</i>	<i>nigra+</i>	37.65	0.04
<i>Parthenocissus</i>	<i>quinquefolia+</i>	16.46	0.42
<i>Phillyrea</i>	<i>angustifolia+</i>	2.67	0.05
<i>Phytolacca</i>	<i>americana+</i>	17.94	0.43
<i>Polygonatum</i>	<i>multiflorum+</i>	0.58	2.93
<i>Prunus</i>	<i>avium+</i>	19.56	0.04
<i>Prunus</i>	<i>domestica+</i>	6.20	0.17
<i>Prunus</i>	<i>laurocerasus+</i>	25.36	0.10
<i>Prunus</i>	<i>mahaleb+</i>	15.83	0.27
<i>Prunus</i>	<i>sp.</i>	1.10	0.41
<i>Prunus</i>	<i>yendoensis</i>	22.21	0.03
<i>Pyracantha</i>	<i>coccinea+</i>	0.30	0.68
<i>Rhamnus</i>	<i>japonicus</i>	6.27	0.97
<i>Rosa</i>	<i>corymbifera</i>	0.94	1.09
<i>Rosa</i>	<i>glauca+</i>	0.46	2.22
<i>Rosa</i>	<i>pimpinellifolia</i>	39.21	0.05
<i>Rosa</i>	<i>rubigenosa</i>	1.14	1.08
<i>Rosa</i>	<i>vil+</i>	0.08	1.23
<i>Sambucus</i>	<i>canadensis+</i>	39.61	0.35
<i>Sambucus</i>	<i>racemosa+</i>	5.53	0.25
<i>Sorbus</i>	<i>aria+</i>	0.50	0.38
<i>Sorbus</i>	<i>aucuparia+</i>	1.02	1.26
<i>Streptopus</i>	<i>amplexifolius</i>	3.29	3.05
<i>Tamus</i>	<i>communis+</i>	1.29	2.71
<i>Taxus</i>	<i>baccata</i>	1.38	0.41
<i>Viburnum</i>	<i>lantana+</i>	1.85	0.19
<i>Viburnum</i>	<i>carlesii</i>	2.41	0.18
<i>Viburnum</i>	<i>opulus+</i>	2.92	1.36

The symbol '+' indicates those species that were used for assessing the 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%),

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

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

STATISTICAL ANALYSES

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

To determine the relationship between fruit colour and pigment composition, we entered the first three PCs as independent variables into multiple regressions with anthocyanin and carotenoid concentrations as dependent variables. Multiple regressions are suitable because all explanatory variables were included in the analysis. Regressions were performed separately for anthocyanins and carotenoids as stepwise backwards and forward procedures, which yielded identical results.

To study whether birds perceive fruits differently depending on their pigment composition, we entered ln-transformed cone excitation values as independent variables in stepwise backwards (and forward) multiple regressions, with anthocyanin or carotenoid contents as dependent variables. To compare birds' food choice of alternative foods and the pigment composition of ripe and unripe fruits of the same species, we used paired *t*-tests. To compare whether fruits originating from the Botanical Gardens differ from fruits collected in Europe, we used a nonparametric Mann–Whitney test. Moreover, to assess whether birds might use pigment concentration 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 contents 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 characterised by positive coefficients from 300–430 nm and above 615 nm and negative coefficients from 435–610 nm. It represented thus the amount of variation in mid-wavelengths relative 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

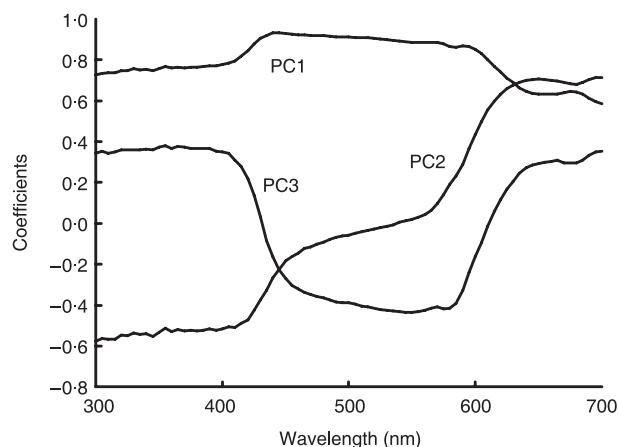


Fig. 1. Principal component (PC) coefficients relating PCs to the original reflectance spectra of 60 fruit species. High positive values at a given wavelength indicate a strong association between the specific PC and that wavelength (e.g. across the entire range for PC1). Likewise, negative values of -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.

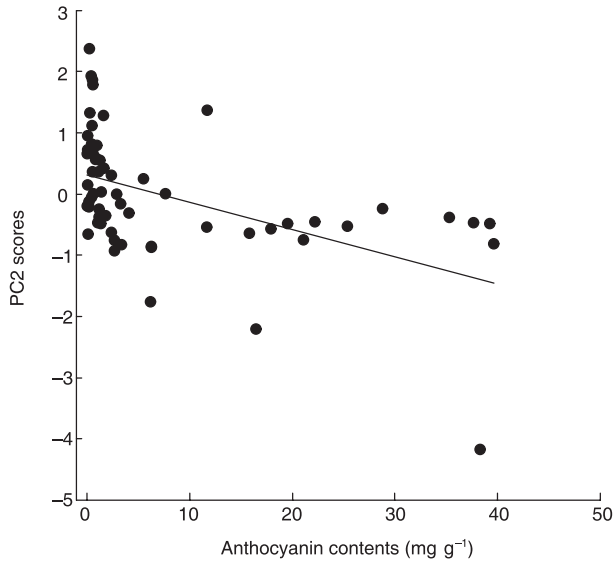


Fig. 2. There is a negative relationship between anthocyanin contents in fruits and the relative amount of short- to long-wavelength reflectance (PC2). Black fruits and fruits reflecting UV have negative PC2 scores, whereas red and orange fruits have positive PC2 scores. Note the extensive variation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

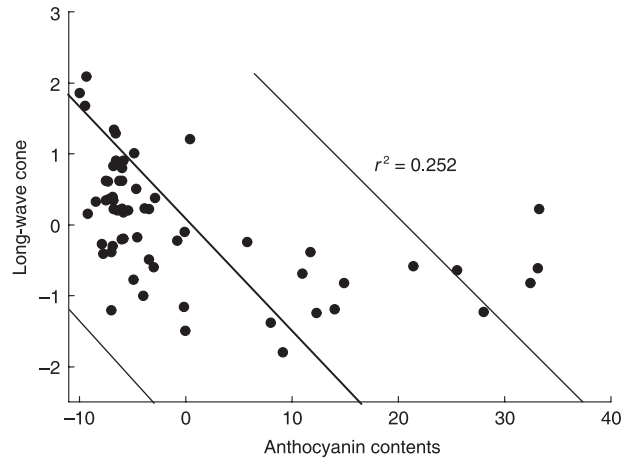


Fig. 3. Partial regression plot for the relationship between anthocyanin contents and long-wave cone outputs, the linear regression is shown with 95% CIs.

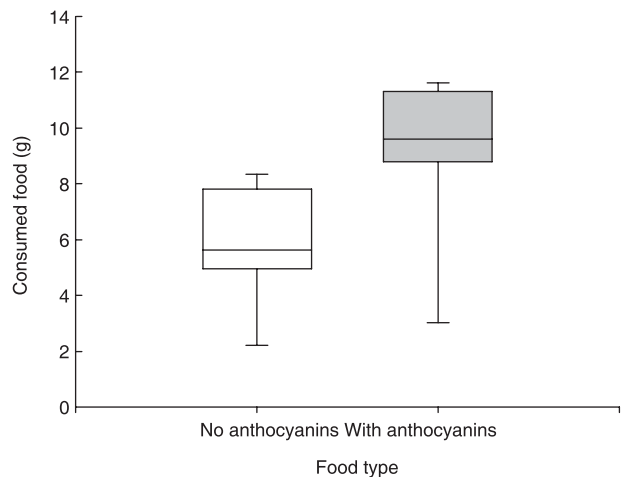


Fig. 4. Birds selected food with anthocyanins over food of identical nutrient composition without anthocyanins. Birds consumed on average 9.6 g (9.1–11.1, 2nd and 3rd quartile) of food with anthocyanins and 5.6 g (5.0–6.7) of control food per day. Illustrated is the mean food intake during the four consecutive days of the experiment, whiskers indicate 90th and 10th percentile.

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

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

Discussion

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

FRUIT COLOUR AS HONEST SIGNAL OF ANTHOCYANINS

Although our study shows that anthocyanin concentrations increase during fruit ripening, we found no relationship between anthocyanin contents and the contrasts between fruits and their background. This result challenges the prevailing notion that the increase in fruit pigmentation during ripening is an adaptation to facilitate detection by diurnal seed dispersers (e.g. Kerner 1895; Schmidt *et al.* 2004). The result that increasing anthocyanin concentrations do not concomitantly increase conspicuousness appears counterintuitive, but it is supported by an experiment documenting that some birds can detect red fruits that have typically low contents of anthocyanins from a larger distance than black fruits that are characterised by high contents of anthocyanins (Schaefer *et al.* 2006).

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

Owing to the consistent correlation between colour and anthocyanin contents, we suggest that blackcaps used the colour differences between the foods as a proximate cue to select anthocyanins in our food choice experiment. Food selection for anthocyanins is not explicable by innate colour preference because blackcaps innately prefer red, not blue food items (Schmidt & Schaefer 2004). Since the food containing anthocyanins was less contrasting than the alternative food, we can exclude that selection for anthocyanins is biased by preferences to consume more conspicuous food (see Schmidt *et al.* 2004). At the evolutionary level, birds might have selected anthocyanins because they indicate high caloric rewards, as shown by the correlation between anthocyanin concentrations and energy contents in fruits. However, we 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 alternative foods (Schaefer *et al.* 2003b).

We thus hypothesise that, ultimately, birds choose anthocyanins 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 (Carlo 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 evidence that birds choose food on the basis of anthocyanins. Thus, we suggest a new hypothesis, which predicts that selection 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 simultaneously 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 constitute a potential reward, the signal is honest because of the unavoidable connection between signal intensity and quality. Our hypothesis that birds preferentially consume fruits containing 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 contents) 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 carotenoids (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

interspecific comparisons of ripe fruits. This result is supported by a previous study on grocery-sold fruits which found no relationship between carotenoids and colour (Lancaster *et al.* 1997). Moreover, carotenoid contents did not differ between ripe and unripe fruits in our study, further corroborating that carotenoid contents are not directly linked to fruit colouration. Consequently, birds aiming to optimize carotenoid intake for coloration or health purposes must rely on tactics other than visual assessment of fruit colour. An influential hypothesis suggests that sensory biases for orange and red nuptial colouration may have evolved in a foraging context to optimise carotenoid intake from fruits (Rodd *et al.* 2002). Given the lack of a relationship between fruit colour and carotenoid contents, our results suggest that sensory biases are unlikely to evolve for that reason, at least in animals that consume a variety of differently coloured fruits.

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

Conclusions

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

Acknowledgements

We thank N. Hart for providing cone sensitivities of the blue tit. We also thank N. Thomas for assistance with pigment analyses and Graeme Ruxton and two anonymous referees for many valuable comments that improved an earlier version. We thank Ulrike Strauss for help in bird maintenance. Birds were kept under license of the district government Weser-Ems, Oldenburg, Germany, and the experiment complied with current laws on animal health. H.M.S. was supported by a DFG grant (Scha 1008/4-1), K.J.M. by the School of Life Sciences and the College of Liberal Arts and Sciences and C.C. by a DAAD grant.

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Received 8 August 2007; accepted 17 October 2007

Handling Editor: Craig Benkman