

# Females of carotenoid-supplemented males are more faithful and produce higher quality offspring

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Reproduction is known to increase the basal metabolic rate and generate oxidative stress, a possible proximate cost of reproduction. Carotenoids have been shown to be *in vitro* antioxidant molecules and, in a number of instances, to contribute *in vivo* to the antioxidant protection of the organism against the deleterious effects of free radicals and oxidative stress. These compounds are also involved in the up- and downregulation of the immune system. Thus, carotenoids may improve a male's health status and condition during breeding and enhance his attractiveness through, for example, a higher investment into mating activities. The differential allocation hypothesis predicts that females should invest more in reproduction when mated to more attractive partners. Therefore, a supplementary dose of carotenoids during breeding should increase male attractiveness and translate into higher reproductive success via a higher reproductive effort by their mate. We tested this hypothesis in great tits by supplementing males with carotenoids during their female's fertile period. We subsequently transferred entire clutches into unmanipulated foster nests. Thus, any effect of our carotenoid supplementation to males on their reproductive success must be due to female differential reproductive investment. Offspring sired by carotenoid-supplemented males were found to grow bigger and heavier and to fledge more successfully. Carotenoid-supplemented males also lost less paternity. Our results illustrate the fitness benefits males can accrue from carotenoids and underline the selective pressure imposed on males to optimize carotenoid acquisition. *Key words*: carotenoids, differential allocation hypothesis, extrapair paternity, great tit, *Parus major*. [*Behav Ecol* 19:1165–1172 (2008)]

During reproduction, birds have to face a steep increase in their metabolic rate, which generates oxidative stress that is hypothesized to be a proximate cost of reproduction (Nilsson 2002; Alonso-Alvarez, Bertrand, Devevey, Prost, et al. 2004; Wiersma et al. 2004). In males, basal metabolic rate increases already long before the egg-laying period when they engage in breeding and mating activities and spend an increasing time in territorial defense, mate guarding, or nest building (Chastel et al. 2003). This increase in reproductive activities is both concomitant with and induced by an increase in testosterone level (Chastel et al. 2003), which itself has been shown to generate oxidative stress (von Schantz et al. 1999; Alonso-Alvarez et al. 2007). Oxidative stress affects overall body condition and provokes muscular fatigue that drives individuals to reduce their activity (Halliwell and Gutteringe 2007).

Carotenoids are a large family of molecules that are part of the antioxidant barriers of vertebrates protecting DNA, proteins, and biological membranes against oxidative stress (Krinsky 2001; Surai 2002; Kiokias and Gordon 2004; Halliwell and Gutteringe 2007). Although their *in vivo* antioxidant property is still debated (Isaksson and Andersson 2008), studies have shown that carotenoids do participate in an individual's antioxidant system (Surai 2002; Alonso-Alvarez, Bertrand, Devevey, Gaillard, et al. 2004; Hórak et al. 2007). A short-term supplementation of carotenoids may thus be expected to temporarily boost a male's antioxidant system, enhance his body condition, and help him to sustain full breeding activity such

as territorial singing and fighting, nest building, mate guarding, etc.

Carotenoids, and particularly  $\beta$ -carotene, have also been shown to be immunoenhancers. Carotenoids and their breakdown and derived products (retinoids, vitamin A) act as hormones and regulate the expression of genes involved in immune cell proliferation and differentiation (Bendich 1989; Chew and Park 2004; Hartley and Kennedy 2004). On the other end of the immune activity, they have also been shown to downregulate the immune system, keeping the inflammatory response (fever, anemia, reduced activity [i.e., sickness behavior], production of reactive oxygen and nitrogen species [RONS]) below deleterious levels (Chew and Park 2004; Koutsos et al. 2006), or alleviating the costs of both cellular and humoral immunity in terms of oxidative damage or body condition (Hórak et al. 2006, 2007). For these reasons, a short-term supplementation of carotenoids may also be expected to help males to better face a pathogen infection, maintain good body condition, and sustain full reproductive activities.

Territorial singing is one of the most obvious mating activity in oscine birds, and song rate is known to be a condition-dependent trait used by female to choose their mate (Duffy and Ball 2002; Ward et al. 2003; Garamszegi et al. 2004; Nowicki and Searcy 2004). Nevertheless, other breeding activities, such as territorial defense, mate guarding, or nest building, are likely to honestly signal male quality and thus to be used by females for mate choice (Lotem et al. 1999). Females do not only exert choice of mate but also can adjust their reproductive investment according to their mate's phenotypic quality. A number of studies in various taxa have now corroborated the differential allocation hypothesis (DAH) (Burley 1986, 1988; Sheldon 2000). Females mated with more attractive males

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Received 28 March 2008; revised 24 June 2008; accepted 25 June 2008.

feed their offspring more (Burley 1988; de Lope and Møller 1993; Limbourg et al. 2004), lay more eggs (Petric and Williams 1993), lay larger eggs, leading to offspring of better condition and competitive ability (Cunningham and Russell 2000; Velando et al. 2006), deposit more antioxidants into their eggs (Williamson et al. 2006), and grant more paternity to their social mate (Safran et al. 2005). Although differential allocation has been mainly tested by altering fixed morphological traits (i.e., male coloration and ornaments), variations in more flexible, behavioral traits may also influence female reproductive investment. For example, Szentirmai and colleagues (Szentirmai et al. 2005) have found female penduline tits to adjust their parental care to the rate at which their mates built the nest.

In this study, we investigated whether supplementing male great tits with a dose of carotenoids during their mate's fertile period would translate into higher male fitness via female differential investment in reproduction. Studies manipulating carotenoid availability have generally supplemented individuals during periods of several weeks. However, such protocols do not allow disentangling direct effects of carotenoids on an animal's health from modifications in life-history traits in response to variations in resource availability (McGraw et al. 2005; Bertrand et al. 2006; Berthouly et al. 2008). Therefore, we chose to provide males with a single physiological dose to favor short-term, direct effects of carotenoids on male health and condition over individual adjustment in reproductive strategies. Male great tits exhibit no carotenoid-pigmented skin or bill parts, and their plumage is molted long before the breeding season. Hence, any effect of our carotenoid supplementation should be due to modifications in some flexible behavioral traits in ways that increase their attractiveness. We tested whether females mated to carotenoid-supplemented males were more faithful than females mated with placebo-treated males. A fostering protocol further enabled us to remove postlaying effects of our treatment on nestling rearing conditions and to examine whether the quality of the offspring varied according to the treatment received by their sire.

## MATERIALS AND METHODS

### Experimental procedure

This experiment was conducted in 2005 on a natural population of great tits *Parus major* breeding in nest-boxes in the Grauholz forest near Berne, Switzerland. Nest-boxes were visited every third day to determine the date when the nest was fully built and the onset of laying. From the laying of the fifth egg onwards, we visited nest-boxes daily to determine incubation date and predict hatching date. We attracted and captured breeding males by playing a song and mimicking an intrusion using a stuffed male, randomly chosen out of 5 different ones, fixed in the middle of a clapnet. The decoy and the loudspeaker were placed within 3 m from the nest-box. At capture, we flipped a coin, and males were then force-fed with either 3 larvae of *Calliphora* spp. alone (placebo) or 3 larvae mixed with a cocktail of carotenoids (carotenoid supplemented). Fresh, living larvae from a commercial fishery shop were mixed with a blend of lutein and zeaxanthin oil and  $\beta$ , $\beta$ -carotene powder (Roche AG, Basel, Switzerland) in the relative proportions found in natural food of great tits (80%, 3%, and 17%, respectively; Partali et al. 1987) to result in a total of 344 mg carotenoids per kilogram of larvae. Most of this blend was ingested by the larvae, and the remaining stuck to their bodies. This mixture was stored at 4 °C in a dark climate chamber to be used within 3 days. The average mass ( $\pm$  standard error [SE]) of a larva was  $91.4 \pm 7.4$  mg ( $n = 100$ ), leading to the supplementation of 0.094 mg of carote-

noids on average. Based on daily energy expenditure (DEE), corresponding daily food intake (DFI), and carotenoid concentration in natural food (Partali et al. 1987; Crocker et al. 2002), this dose approximately doubled the daily carotenoid supply male great tits obtain from their food (0.074 mg; average male body mass: 18.75 g; DEE: 78.91 kJ/day; DFI: 22.15 g of lepidopteran larvae/day). This relatively small dose in comparison to previous studies was chosen for 3 reasons. First, a study by de Ayala and colleagues has shown that providing more than 1 standard deviation of the daily natural supply of an antioxidant such as vitamin E leads to saturation or no effect (de Ayala et al. 2006). Second, at high concentration, autoxidation processes diminish carotenoid antioxidant activity and may even induce oxidative cascades (Kiokias and Gordon 2004), a phenomenon that could partly account for the contradictory results found on antioxidant properties of carotenoids in vivo (Kiokias and Gordon 2004). Finally, carotenoids are lipid-soluble antioxidants that birds can store in their liver (Surai 2002). Doubling the daily dose may lead to part of the extra carotenoids to be stored and used in the following days, thus prolonging the effect of our treatment. Males were weighed ( $\pm 0.1$  g), measured (wing length  $\pm 0.5$  mm and tarsus length  $\pm 0.05$  mm), and individually marked with an aluminum ring for later identification as residents.

Males do not courtship-feed their mates and do not incubate the eggs (Helfenstein F, personal observation; Kluijver 1950; Walker 1977). However, they occasionally feed their mates during incubation. Therefore, to avoid any confounding effect of treatment on reproductive output from the end of the egg laying onwards, we transferred whole clutches to foster nests for eggs to be incubated and nestlings to be raised by unmanipulated parents. We swapped clutches of identical sizes on the day incubation started. We weighed the whole clutch to the nearest 0.01 g. We thereafter checked hatching success in the foster nests and monitored nestling growth by measuring their body mass at 0 (hatching day), 6, 9, and 14 days. We also measured their wing ( $\pm 0.5$  mm), tarsus ( $\pm 0.05$  mm), and sternum length ( $\pm 0.05$  mm) at 6, 9, and 14 days.

Thirty-four males were captured in the vicinity of their nests between -4 and 3 days around the laying of the first egg by their mates (days relative to laying day  $\pm$  SE:  $-0.61 \pm 0.47$ ). Two clutches did not hatch, and eggs showed no sign of development. We captured both parents of the experimental pairs when the nestlings they raised were 12 days old and measured their body mass ( $\pm 0.1$  g), wing length ( $\pm 0.5$  mm), and tarsus length ( $\pm 0.05$  mm). One of the males turned out not to be the same as the one trapped during chick rearing and was excluded from the sample. We failed to recapture 8 other males, and their status as breeders could not be confirmed. We thus conducted all analyses successively including ( $n = 31$ ) or excluding these unconfirmed males ( $n = 23$ ). The full data set allows more statistical power but is potentially more conservative as it may include untreated males and vice versa for the reduced data set. In all cases, we obtain similar results from both data sets, and we only present results from the full data set. Our sample comprises 196 nestlings reared in 31 foster nests.

### Paternity analyses

A blood drop (ca., 2.5  $\mu$ L) was taken from the ulnar vein of both parents from experimental pairs and transferred into 500  $\mu$ L of Tris-ethylenediaminetetraacetic acid (EDTA). A blood drop (ca., 2.5  $\mu$ L) was taken from the tarsal vein of nestlings on their hatching day and transferred into 300  $\mu$ L of Tris-EDTA. When a hatchling died before a blood sample could be taken or when a fertile egg did not hatch, a tissue fragment from the chick or embryo was collected and transferred into 1 mL of

absolute ethanol. Blood and tissue samples were stored at  $-20^{\circ}\text{C}$  until further analyses. DNA was extracted using magnetic beads (MagneSil Blue, Promega, D udendorf, Switzerland). Twenty-three families with 151 nestlings were analyzed for paternity at 11 microsatellite loci (Saladin et al. 2003). We used Cervus 2.0 software package (Marshall et al. 1998; Slate et al. 2000) to calculate allele frequencies, heterozygosity values, exclusion probabilities, and deviation from Hardy–Weinberg equilibrium based on the genetic data of 115 adult great tits (49 females and 66 males) captured in the same study area. Our population did not deviate significantly from Hardy–Weinberg equilibrium at any of the 11 loci. Exclusion power of all loci was 0.99963 for the first parent and 0.999996 for the second parent. Parentage assignment was carried out with Cervus 2.0. All nestlings were assigned to their social mother. Nestlings whose genotype mismatched their social father’s genotype at 2 or more loci were considered as extrapair.

### Predictions and statistical analyses

Males were captured at different times relative to when their mates laid their first egg. Therefore, our treatment should have a higher impact on nestlings hatched from eggs laid after male supplementation in contrast to nestlings hatched from eggs laid before or on the day of supplementation. However, identifying from which egg chicks have hatched would have required marking the eggs as they were laid and artificially incubate the eggs because the hatching of several eggs can occur within few hours. We thus used the nestling mass–based ranks at hatching as a proxy to laying order. Mass-based ranking at hatching shows several advantages. First, it accounts for the number of eggs laid after the treatment as it can be predicted that, even when capture occurred after the laying of the first egg, the treatment should have a stronger impact as females laid larger clutches. Second, first laid eggs are incubated first and are likely to be first hatched. Parents start feeding the chicks as soon as they hatch, and laying order is mirrored and amplified through hatching asynchrony, even among chicks hatched on the same day but at different times. Third, mass-based ranks at hatching account for hatching delay, which may spread over several days (Glutz von Blotzheim and Bauer 1993). All nestlings were weighed on their day of hatching. However, further measures were taken when the first-hatched chick reached the age of 6, 9, and 14 days. Nestling rank includes hatching asynchrony and allows using the age of the first chick as the time factor when investigating chick growth. Mass-based ranking nevertheless imperfectly reflects laying order, and rather than predicting a clear cutoff in the effect our treatment has on nestling growth, we predict an interaction between nestling rank and male treatment. We computed a nestling’s rank based on his body mass on the day of his hatching, accounting for hatching asynchrony, that is, the lighter the nestling and the later he hatched relative to the day of the brood’s first hatching the higher his rank.

The timing of our treatment relative to laying may also affect the effectiveness of our treatment on the number of eggs females laid, the mass of the eggs, the hatchability of these eggs, and fledging success. This factor may also affect females’ ability to adjust their paternity strategy. We thus included the time relative to laying and its interaction with the treatment in models investigating the above-mentioned variables. Similar to nestling growth, we predicted a significant interaction between the treatment and the timing of the treatment relative to the laying of the first egg.

Nestling growth in relation to the treatment of their father was modeled using generalized linear mixed models for repeated measures using the restricted maximum likelihood estimation method (REML–GLMM) and assuming normal

distribution of the error. The starting models comprised the nestling’s rank, the age at measurement, the treatment received by the mother’s mate, and all the possible 2-way and 3-way interactions between age, treatment, and nestling rank. Age was defined as the repeat and nestling identity nested in the nest as the subject. We modeled intra-subject variance using an unstructured  $R$  matrix that accounts for unequal spacing between measures, heteroscedasticity between measurement times (e.g., between ages 0 and 6), and intra-subject correlations between measures (Littell et al. 2006). We further declared the nest nested in the treatment and the interaction between the nest and the repeat (age) as random factors to fully account for the hierarchical structure of our data. Degrees of freedom were computed using Satterthwaite approximation (Littell et al. 2006). Model selection was carried out by removing, one by one, interactions that were the furthest from statistical significance, starting with the highest order interactions.  $F$  and  $P$  values of nonsignificant terms correspond to values just before their removal. We used 2-tailed type 3 tests for fixed effects.

We used  $t$ -test for simple comparisons between groups, applying the Satterthwaite correction when samples had unequal variances. We analyzed clutch size with a generalized linear model with Poisson distribution and a log-link function and egg mass using a general linear model with the mass of the whole clutch as the dependent variable and clutch size as a covariate. Hatching success (i.e., the number of eggs hatched over the number of eggs laid), fledging success (i.e., the number of nestlings fledged over the number of hatchlings), the proportion of extrapair young in the social male’s brood (i.e., the number of extrapair young over the number of nestlings in the brood), and the probability of losing paternity (the presence or absence of extrapair young in the brood) were analyzed with a generalized linear model with binomial distribution and a logit-link function.

Our small sample faces an increased risk of type I error. First, a single carotenoid-supplemented male that would be of higher than average quality and/or would be paired to a higher than average quality female could artificially draw results to significance. To address this problem, we first thoroughly examined the residuals to identify any outlier or group of outliers that would drag the models to significance. Second, we applied a jackknife procedure to determine which experimental nest could induce artificial significant results. All models were first run including all males and then rerun  $N - 1$  times ( $N$  = number of experimental males) each time excluding a different male. If all models lead to qualitatively similar results, the data set is homogeneous and robust. All tests are 2 tailed with a significance level set to  $\alpha = 0.05$ . Analyses were conducted with the SAS software (SAS  2003).

### RESULTS

We found that placebo ( $n = 17$ ) and carotenoid-supplemented ( $n = 14$ ) males did not differ with regard to their body mass, tarsus length, and wing length or with regard to when they were captured relative to egg laying (all  $t < 1.30$ , all  $P > 0.21$ ). Females paired to placebo or carotenoid-supplemented males did also not differ in their body mass or wing length (all  $t < 0.15$ ,  $P > 0.88$ ). However, females paired to carotenoid-supplemented males tended to have longer tarsi ( $t = 2.02$ ,  $P = 0.07$ ). Females from the 2 groups nevertheless laid their first egg at similar dates ( $t = 0.91$ ,  $P = 0.37$ ) and laid clutches of similar sizes (mean clutch size  $\pm$  SE:  $7.29 \pm 0.34$ ; treatment,  $F_{1,30} = 1.28$ ,  $P = 0.27$ ; time of capture relative to laying,  $F_{1,30} = 2.36$ ,  $P = 0.13$ ; time of capture relative to laying  $\times$  treatment,  $F_{1,30} = 0.38$ ,  $P = 0.54$ ). To account for the tendency

**Table 1**  
**REML–GLMM for repeated measures testing the effect of the treatment received by the male with which the female was mated (carotenoid supplemented or placebo) on nestling body mass (log transformed) at 0, 6, 9, and 14 days after hatch**

Type 3 tests of fixed effects				
Effect <sup>a</sup>		Estimate ± SE	$F_{df}$	$P$
Nestling rank		−0.019 ± 0.003	290.5 <sub>1,152</sub>	<0.0001
Age <sup>b</sup>	0	−2.211 ± 0.026	3032.1 <sub>3,111</sub>	<0.0001
	6	−0.485 ± 0.025		
	9	−0.164 ± 0.024		
Treatment <sup>c</sup>		0.031 ± 0.040	0.60 <sub>1,35.1</sub>	0.44
Female tarsus		−0.035 ± 0.031	1.29 <sub>1,20.6</sub>	0.27
Rank × age <sup>b</sup>	0	−0.033 ± 0.003	50.3 <sub>3,142</sub>	<0.0001
	6	−0.026 ± 0.003		
	9	−0.014 ± 0.002		
Rank × treatment <sup>c</sup>		0.008 ± 0.004	5.07 <sub>1,159</sub>	0.026

Age was used as the repeat and the nestling's identity as the subject; the rearing nest and its interaction with age were further declared as random factors (variance/covariance parameter estimates not shown).

<sup>a</sup> The terms rank × treatment × age ( $F_{3,141} = 1.98$ ,  $P = 0.12$ ) and treatment × age ( $F_{3,108} = 1.74$ ,  $P = 0.16$ ) did not reach significance and were backward removed. Their removal did not affect the other terms' significance or the parameter estimates.

<sup>b</sup> Relative to age 14.

<sup>c</sup> Carotenoid supplemented relative to placebo group.

for females paired to carotenoid-supplemented males to have longer tarsi, we included female tarsus length in all subsequent analyses.

In agreement with our prediction that the male treatment should impact more strongly on chicks hatched from eggs further in the laying sequence, we found nestling mass gain and sternum and wing growth to depend both on the treatment received by the male of the genetic mother and on nestling rank (Tables 1 and 2). Last-ranking nestlings produced by females mated with carotenoid-supplemented males grew heavier and grew longer sternum and wings, compared with last-ranking nestlings produced by females mated to placebo males (Figure 1b,d,f). Such differences were not detectable between first-ranking nestlings (Figure 1a,c,e). The treatment did not affect tarsus growth (REML–GLMM: female tarsus length,  $F_{1,20.8} = 2.03$ ,  $P = 0.17$ ; treatment,  $F_{1,49.6} = 1.14$ ,  $P = 0.29$ ; age,  $F_{2,104} = 596.5$ ,  $P < 0.0001$ ; nestling rank,  $F_{1,143} = 87.7$ ,  $P < 0.0001$ ; age × treatment,  $F_{2,104} = 0.00$ ,  $P = 0.99$ ; age × rank,  $F_{2,144} = 27.6$ ,  $P < 0.0001$ ; treatment × rank,  $F_{1,143} = 2.08$ ,  $P = 0.15$ ; age × treatment × rank,  $F_{2,144} = 1.00$ ,  $P = 0.37$ ). Consequently, a higher proportion of offspring hatched from eggs laid by females mated to carotenoid-supplemented males successfully fledged (least-square means [95% confidence limits]; carotenoid-supplemented males: 0.94 [0.84, 0.98]; placebo males: 0.67 [0.56, 0.77]); female tarsus length,  $F_{1,21} = 4.98$ ,  $P = 0.04$ ; treatment,  $F_{1,21} = 10.84$ ,  $P = 0.004$ ; time of capture relative to laying,  $F_{1,19} = 0.26$ ,  $P = 0.62$ ; time of capture relative to laying × treatment,  $F_{1,19} = 0.43$ ,  $P = 0.52$ ; Figure 2). Females mated with carotenoid-supplemented males did not lay heavier eggs compared with females mated to placebo males (analysis of covariance on the mass of the whole clutch corrected for clutch size: clutch size,  $F_{1,26} = 34.1$ ,  $P < 0.0001$ ; female tarsus length,  $F_{1,23} = 3.89$ ,  $P = 0.06$ ; treatment,  $F_{1,26} = 0.03$ ,  $P = 0.86$ ; time of capture relative to laying,  $F_{1,26} = 1.06$ ,  $P = 0.31$ ; time of capture relative to laying × treatment,  $F_{1,26} = 0.53$ ,  $P = 0.47$ ). Hatching success was not affected by our treatment. However, the timing of capture relative to egg laying impacted on egg hatchability with earlier captures leading to lower hatching success (female tarsus length,  $F_{1,21} = 0.10$ ,  $P = 0.76$ ; treatment,  $F_{1,27} = 0.01$ ,  $P =$

0.91; time of capture relative to laying,  $F_{1,27} = 6.04$ ,  $P = 0.02$ ; time of capture relative to laying × treatment,  $F_{1,27} = 1.40$ ,  $P = 0.25$ ). This suggests that trapping was indeed a stress for the birds.

Carotenoid-supplemented males had a lower proportion of extrapair young in their brood (least-square means [95% confidence limits]; carotenoid-supplemented males: 0.046 [0.01, 0.14]; placebo males: 0.163 [0.10, 0.26]; treatment,  $F_{1,21} = 4.45$ ,  $P = 0.047$ ; female tarsus length,  $F_{1,18} = 0.17$ ,  $P = 0.68$ ; time of capture relative to laying,  $F_{1,19} = 2.75$ ,  $P = 0.11$ ; time of capture relative to laying × treatment,  $F_{1,19} = 2.51$ ,  $P = 0.13$ ). We also found that carotenoid-supplemented males had a lower chance to lose paternity (at least one extrapair young in the brood) than placebo males (least-square means [95% confidence limits]; carotenoid-supplemented males: 0.20 [0.05, 0.56]; placebo males: 0.69 [0.39, 0.89]; treatment,  $F_{1,21} = 4.90$ ,  $P = 0.038$ ; female tarsus length,  $F_{1,18} = 0.12$ ,  $P = 0.73$ ; time of capture relative to laying,  $F_{1,19} = 0.0$ ,  $P = 0.95$ ; time of capture relative to laying × treatment,  $F_{1,19} = 0.0$ ,  $P = 0.95$ ; Figure 3).

Our small sample renders our analyses vulnerable to type I error. An untypical experimental male or pair of foster parents may create misleading significant results. However, residuals of all the REML–GLMMs satisfied modeling assumptions, and plotting studentized residuals against predicted values did not reveal outliers that would drag results to significance. The jackknife procedures confirmed the analysis of the residuals. For all analysis, all the  $N - 1$  models generated results qualitatively similar (i.e., sign, magnitude, and significance of effects) to the model built on the complete data set. Eventually, we could not identify outliers that would have produced fallacious significant results.

## DISCUSSION

Our results suggest that fertile females mated to carotenoid-supplemented males perceived a change in their mate's phenotype and adjusted their reproductive investment accordingly. Consequently, carotenoid-supplemented males sired more offspring of better quality.

**Table 2**  
**REML–GLMM for repeated measures testing the effect of the treatment received by the male with which the female was mated (carotenoid supplemented or placebo) on nestling sternum length and wing length at 6, 9, and 14 days after hatch**

Sternum length		Wing length	
Type 3 tests of fixed effects			
Effect	Estimate ± SE	<i>F</i> <sub>df</sub>	<i>P</i>
Nestling rank	-0.024 ± 0.049	88.8 <sub>1,158</sub>	<0.0001
Age <sup>a</sup>	-4.868 ± 0.290	371.5 <sub>2,109</sub>	<0.0001
Treatment <sup>b</sup>	0.287 ± 0.281		
Female tarsus	0.113 ± 0.264	4.99 <sub>1,37.6</sub>	0.03
Treatment × age <sup>c</sup>	-0.374 ± 0.416	3.1 <sub>3,2,109</sub>	0.07
Carotenoid supplementation at 6	0.653 ± 0.401		
Carotenoid supplementation at 9	-0.073 ± 0.054	2.05 <sub>2,155</sub>	0.13
Rank × age <sup>a</sup>	-0.038 ± 0.052		
Rank × treatment <sup>b</sup>	0.154 ± 0.065	7.81 <sub>1,58</sub>	0.38
Rank × treatment × age <sup>c</sup>	-0.185 ± 0.073	8.3 <sub>4,2,155</sub>	0.0004
Carotenoid supplementation at 6			
Carotenoid supplementation at 9			

Type 3 tests of fixed effects			
Effect	Estimate ± SE	<i>F</i> <sub>df</sub>	<i>P</i>
Nestling rank	-0.937 ± 0.107	160.1 <sub>1,139</sub>	<0.0001
Age <sup>a</sup>	-32.44 ± 0.64	2609.2 <sub>2,78.8</sub>	<0.0001
Treatment <sup>b</sup>	0.970 ± 1.173		
Female tarsus	-0.986 ± 0.303	0.56 <sub>1,41.7</sub>	0.46
Treatment × age <sup>c</sup>	-0.415 ± 0.927	1.95 <sub>1,20.9</sub>	0.18
Carotenoid supplementation at 6	-0.279 ± 0.871	0.10 <sub>2,78.8</sub>	0.90
Carotenoid supplementation at 9	0.408 ± 0.088	22.2 <sub>2,136</sub>	<0.0001
Rank × age <sup>a</sup>	-0.098 ± 0.069		
Rank × treatment <sup>b</sup>	0.328 ± 0.153	1.05 <sub>1,139</sub>	0.31
Rank × treatment × age <sup>c</sup>	-0.303 ± 0.123	6.17 <sub>2,136</sub>	0.003
Carotenoid supplementation at 6			
Carotenoid supplementation at 9			

Age was used as the repeat and the nestling's identity as the subject; the rearing nest and its interaction with age were further declared as random factors (variance/covariance parameter estimates not shown).

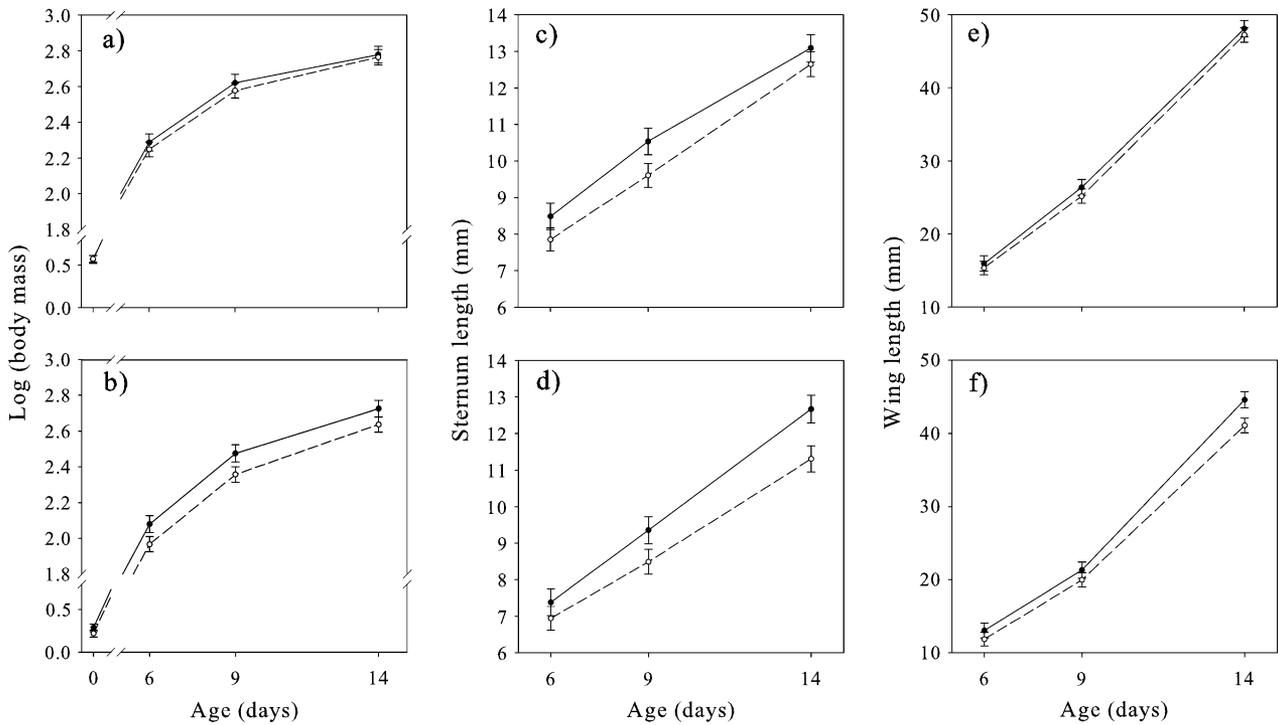
<sup>a</sup> Relative to age 14.  
<sup>b</sup> Carotenoid supplemented relative to placebo group.  
<sup>c</sup> Relative to all other groups.

Carotenoids have numerous physiological properties. For instance, they participate in an individual's antioxidant system (Krinsky 2001; Alonso-Alvarez, Bertrand, Devevey, Gaillard, et al. 2004; Kiokias and Gordon 2004; Halliwell and Guttering 2007; Hōrak et al. 2007). Supplementary carotenoids may have boosted males' antioxidant system and thus helped them to maintain high levels of costly activities such as territorial singing (Ward et al. 2003; Barnett and Briskie 2007) or mate guarding (Komdeur 2001; Low 2006). Carotenoids, and especially β-carotene, are also known immunoenhancers. Carotenoids and their derivative products act as hormones to regulate the expression of genes involved in immune cell activity (Bendich 1989; Chew and Park 2004; Hartley and Kennedy 2004). They also alleviate the cost of immunity by downregulating the innate (fever, anemia, reduced activity, i.e., sickness behavior [Koutsos et al. 2006]), cellular, and humoral immune responses (production of oxidative molecules [Chew and Park 2004; Hōrak et al. 2006, 2007]), which translates into better body condition (Hōrak et al. 2006, 2007). Carotenoids act as hormones in these circumstances and these effects could potentially be rapidly triggered. A supplementary dose of carotenoids may have thus helped males to better face a pathogen infection and to maintain good body condition and high levels of mating activity.

A recent paper by Delhey and colleagues (Delhey et al. 2007), reviewing the various "cosmetics," for example, pigmented preening oil, birds use to alter the color of their plumage provides another mechanism by which male attractiveness could rapidly change. Whether the uropygial oil of great tits contains carotenoids or any other pigment is currently unknown. Nevertheless, if carotenoids were to be present in the preening oil, they could contribute to the plumage coloration when deposited with the oil on the feathers and then increase male attractiveness.

In response to the change in their mate's phenotype, for example, their mating activities or cosmetic coloration, females appear to have adjusted their reproductive strategies. The better growth and fledging success of nestlings sired by supplemented males cannot be attributed to greater parental care by the experimental males or females because all clutches were moved to foster nests at the start of incubation. All the eggs were therefore incubated and the nestlings raised, by unmanipulated birds. Consequently, effects measured on the nestlings must have arisen via differential investment into the eggs. Male great tits do not courtship-feed their mate (Walker 1977) and are thus unlikely to directly influence egg quality. As females mated to both types of males laid clutches of similar mass, their eggs must have differed in their composition. Females may have deposited different amounts of nutrients (e.g., lipids [Royle et al. 1999]), antioxidants (e.g., carotenoids and vitamins A and E [Blount et al. 2000; Royle et al. 2001; Biard et al. 2005; Karadas et al. 2005; Williamson et al. 2006]), or hormones (e.g., testosterone [Gil et al. 1999; Royle et al. 2001]) into the eggs. These results are in accordance with the DAH (Burley 1986, 1988), which proposes that females should adjust their reproductive investment to male attractiveness.

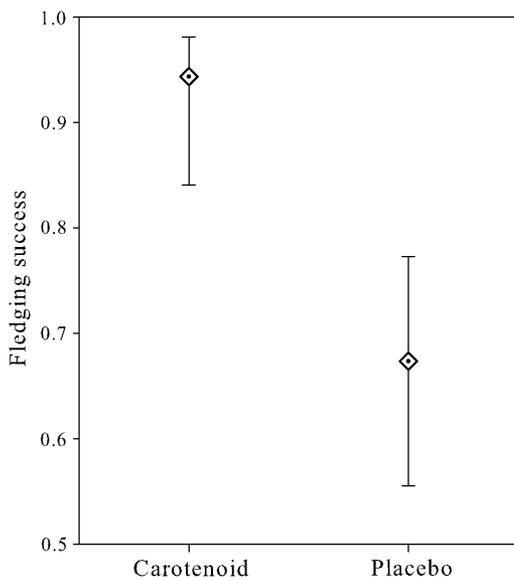
Females not only laid eggs that produced offspring of better quality but also granted more paternity to their mates, which is also in accordance with the DAH. If supplementary carotenoids affected mate-guarding behavior, males may have been better able to secure their paternity. However, female birds have been shown to retain most of the control over copulation and fertilization (Cunningham and Birkhead 1997; Michl et al. 2002), and paternity assurance strategies are generally regarded as the best-of-a-bad-job (Johnsen et al. 1998). Thus, the higher paternity achieved by carotenoid-supplemented males is likely to reflect female fertilization strategies.



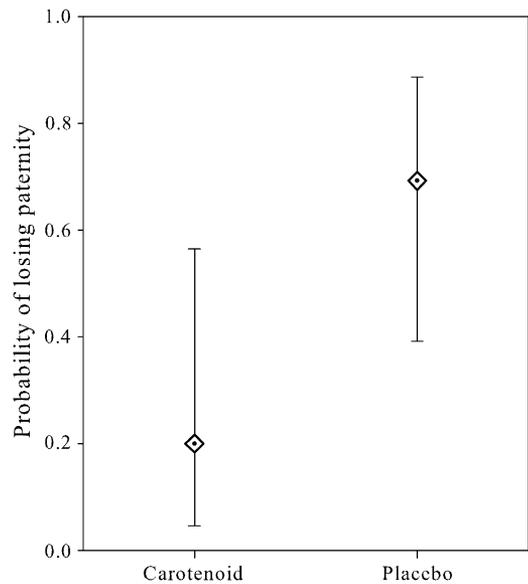
**Figure 1** Nestling growth in relation to the treatment received by the male with which their mother was paired (carotenoid-supplemented males: black dots and solid line; placebo males: open circles and dashed line) and their mass-based rank at hatching. Body mass at days 0, 6, 9, and 14 after hatch of (a) first-hatched nestlings and (b) nestlings of rank 7 (the average brood size at hatching [range 4–10]). Sternum length at days 6, 9, and 14 after hatch of (c) first-hatched nestlings and (d) nestlings of rank 7. Left wing length at days 6, 9, and 14 after hatch of (e) first-hatched nestlings and (f) nestlings of rank 7. Values are least-square means  $\pm$  SE from REML–GLMMs.

An alternative to the DAH could be that carotenoid-supplemented males produced sperm of better quality. Vertebrate sperm show high rates of metabolic activity and free radical production and are rich in polyunsaturated fatty acids, which makes them particularly susceptible to oxidative stress (Surai et al. 1998; Brèque et al. 2003) that lead to extensive cellular

damage (Twigg et al. 1998). Sperm also contain highly condensed nucleic DNA and reduced cytoplasm resulting in DNA damage that is more likely to accumulate without repair (Vishwanath and Shannon 1997). These oxidative damages to sperm membranes and sperm DNA can lead to increased infertility, offspring of lower quality (reviewed in Blount et al.



**Figure 2** Proportion of successfully fledged offspring (least-square means  $\pm$  95% confidence limits) in relation to the treatment received by the male with which the mother was paired.



**Figure 3** Probability (least-square means  $\pm$  95% confidence limits) that the male's social brood contained at least one extrapair offspring in relation to the treatment he received.

2001), and reduced sperm swimming ability (Møller et al. 2008), a prominent parameter of sperm competitive ability (Birkhead et al. 1999; Denk et al. 2005). The supplemental dose of carotenoids may have enhanced males' antioxidant protection and thus enabled them to produce better sperm with higher competitive ability leading to higher paternity and better offspring quality. This hypothesis has received some support from correlational studies where either males displaying more carotenoid-based colors produce faster and more viable sperm (Peters et al. 2004; Locatello et al. 2006) or males with higher levels of plasma carotenoids, vitamin A, and vitamin E produced less abnormal sperm (Møller et al. 2005). However, although the avian semen contains a set of antioxidant molecules (Surai et al. 1998; Brèque et al. 2003), it has not been found to contain carotenoids or derivative products (e.g., vitamin A). Therefore, any effect of carotenoids on antioxidant protection of sperm must be indirect, via a contribution to the overall antioxidant system ultimately reducing oxidative stress in the testes. However, oxidative stress is supposed to mainly result in damage to sperm membrane, cytoskeleton, and axoneme, which should translate into greater occurrence of infertile eggs due to impaired sperm-egg interactions (Blount et al. 2001). We did not find our treatment to affect hatching success. Hence, although an indirect impact of carotenoid on sperm quality is still possible, the higher paternity of carotenoid-supplemented males and the better quality of the offspring they sired are likely to be mostly due to female reproductive decisions.

Whatever the underlying mechanism that led to carotenoid-supplemented males to have higher paternity and higher quality offspring, the effect of the supplementation may have been amplified by the experimental procedure itself. We captured and retained males till the time needed to force-feed them and collect various measures. This procedure may have generated a short-term acute stress response characterized by a transient acceleration of the metabolism and an elevation of plasma corticosterone (Buchanan 2000), 2 processes likely to generate oxidative stress (Lin et al. 2004; Halliwell and Gutteridge 2007). In this context, a supplemental dose of carotenoids may have been critical for males to face this stress-induced oxidative stress and recover from capture. In addition, the temporary male retention may have created a situation favorable to the pursuit of extra-pair copulations by females. The supplemental dose of carotenoids may have enabled males to recover quicker and either better guard their females or better protect their sperm against the stress-induced oxidative stress.

To conclude, we found that carotenoid-supplemented males enjoyed higher paternity, sired higher quality offspring, and had a higher reproductive success. Our results suggest that this higher reproductive success arises through female differential allocation into the eggs and choice of paternity, although paternal effects, via an indirect protection against oxidative stress offered by carotenoids to sperm, cannot be fully discarded. These results illustrate the fitness benefits males can accrue from carotenoids and underline the selective pressure imposed on males to optimize the acquisition of these compounds.

## FUNDING

Swiss National Science Foundation (3100A0-102017 to H.R.).

The authors thank L. Badey for her help with fieldwork and Roche Vitamins Inc. for donating the carotenoids. This work was conducted under license of the Ethical Committee of the Agricultural Office of the Canton Bern and a ringing permit provided by the Agency for Environment, Forests, and Landscapes.

## REFERENCES

- Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G. 2004a. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and anti-oxidant activity. *Am Nat.* 164:651–659.
- Alonso-Alvarez C, Bertrand S, Devevey G, Prost J, Faivre B, Sorci G. 2004b. Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol Lett.* 7:363–368.
- Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proc R Soc Lond B Biol Sci.* 274:819–825.
- Barnett C, Briskie JV. 2007. Energetic state and the performance of dawn chorus in silvereyes (*Zosterops lateralis*). *Behav Ecol Sociobiol.* 61:579–587.
- Bendich A. 1989. Carotenoids and the immune-response. *J Nutr.* 119:112–115.
- Berthouly A, Helfenstein F, Tanner M, Richner H. 2008. Sex-related effects of maternal egg investment on offspring in relation to carotenoid availability in the great tit. *J Anim Ecol.* 77:74–82.
- Bertrand S, Alonso-Alvarez C, Devevey G, Faivre B, Prost J, Sorci G. 2006. Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. *Oecologia.* 147:576–584.
- Biard C, Surai PF, Møller AP. 2005. Effects of carotenoid availability during laying on reproduction in the blue tit. *Oecologia.* 144:32–44.
- Birkhead TR, Martinez JG, Burke T, Froman DP. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proc R Soc Lond B Biol Sci.* 266:1759–1764.
- Blount JD, Houston DC, Møller AP. 2000. Why egg yolk is yellow. *Trends Ecol Evol.* 15:47–49.
- Blount JD, Møller AP, Houston DC. 2001. Antioxidants, showy males and sperm quality. *Ecol Lett.* 4:393–396.
- Brèque C, Surai PF, Brillard J-P. 2003. Roles of antioxidants on prolonged storage of avian spermatozoa in vivo and in vitro. *Mol Reprod Dev.* 66:314–323.
- Buchanan KL. 2000. Stress and the evolution of condition-dependent signals. *Trends Ecol Evol.* 15:156–160.
- Burley NT. 1986. Sexual selection for aesthetic traits in species with biparental care. *Am Nat.* 127:415–445.
- Burley NT. 1988. The differential-allocation hypothesis: an experimental test. *Am Nat.* 132:611–628.
- Chastel O, Lacroix A, Kersten M. 2003. Pre-breeding energy requirements: thyroid hormone, metabolism and the timing of reproduction in house sparrows *Passer domesticus*. *J Avian Biol.* 34:298–306.
- Chew BP, Park JS. 2004. Carotenoid action on the immune response. *J Nutr.* 134:257S–261S.
- Crocker D, Hart A, Gurney J, McCoy C. 2002. Project PN0908: method for estimating daily food intake of wild birds and mammals. York (UK): Central Science Laboratory.
- Cunningham EJA, Birkhead TR. 1997. Female roles in perspective. *Trends Ecol Evol.* 12:337–338.
- Cunningham EJA, Russell AF. 2000. Egg investment is influenced by male attractiveness in the mallard. *Nature.* 404:74–77.
- de Ayala RM, Martinelli R, Saino N. 2006. Vitamin E supplementation enhances growth and condition of nestling barn swallows (*Hirundo rustica*). *Behav Ecol Sociobiol.* 60:619–630.
- de Lope F, Møller AP. 1993. Female reproductive effort depends on the degree of ornamentation of their mates. *Evolution.* 47:1152–1160.
- Delhey K, Peters A, Kempenaers B. 2007. Cosmetic coloration in birds: occurrence, function and evolution. *Am Nat.* 169:145–158.
- Denk AG, Holzmann A, Peters A, Vermeirssen ELM, Kempenaers B. 2005. Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance. *Behav Ecol.* 16:825–833.
- Duffy DL, Ball GF. 2002. Song predicts immunocompetence in male European starlings (*Sturnus vulgaris*). *Proc R Soc Lond B Biol Sci.* 269:847–852.
- Garamszegi LZ, Møller AP, Török J, Michl G, Péczely P, Richard M. 2004. Immune challenge mediates vocal communication in a passerine bird: an experiment. *Behav Ecol.* 15:148–157.
- Gil D, Graves J, Hazon N, Wells A. 1999. Male attractiveness and differential testosterone investment in zebra finch eggs. *Science.* 286:126–128.

- Glutz von Blotzheim UN, Bauer KM. 1993. Handbuch der Vögel Mitteleuropas, Passeriformes. Wiesbaden (Germany): Aula.
- Halliwell B, Puttering JMC. 2007. Free radicals in biology and medicine. 4th ed. Oxford: Oxford University Press.
- Hartley RC, Kennedy MW. 2004. Are carotenoids a red herring in sexual display? *Trends Ecol Evol.* 19:353–354.
- Hörak P, Saks L, Zilmer M, Karu U, Zilmer K. 2007. Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *Am Nat.* 170:625–635.
- Hörak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K. 2006. Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. *J Exp Biol.* 209:4329–4338.
- Isaksson C, Andersson S. 2008. Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. *Proc R Soc Lond B Biol Sci.* 275:309–314.
- Johnsen A, Lifjeld JT, Rohde PA, Primmer CR, Ellegren H. 1998. Sexual conflict over fertilizations: female bluethroats escape male paternity guards. *Behav Ecol Sociobiol.* 43:401–408.
- Karadas F, Pappas AC, Surai PF, Speake BK. 2005. Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. *Comp Biochem Physiol Part B Biochem Mol Biol.* 141:244–251.
- Kiokias S, Gordon MH. 2004. Antioxidant properties of carotenoids in vitro and in vivo. *Food Rev Int.* 20:99–121.
- Kluijver HN. 1950. Daily routine of the great tit, *Parus m. major* L. *Ardea.* 38:99–135.
- Komdeur J. 2001. Mate guarding in the Seychelles warbler is energetically costly and adjusted to paternity risk. *Proc R Soc Lond B Biol Sci.* 268:2103–2111.
- Koutsos EA, Lopez JCG, Klasing KC. 2006. Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (*Gallus gallus domesticus*). *J Nutr.* 136:1027–1031.
- Krinsky NI. 2001. Carotenoids as antioxidants. *Nutrition.* 17:815–817.
- Limbourg T, Mateman AC, Andersson S, Lessells CM. 2004. Female blue tits adjust parental effort to manipulated male UV attractiveness. *Proc R Soc Lond B Biol Sci.* 271:1903–1908.
- Lin H, Decuyper E, Buysse J. 2004. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) 2. Short-term effect. *Comp Biochem Physiol Part B Biochem Mol Biol.* 139:745–751.
- Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. 2006. SAS for mixed models. 2nd ed. Cary (NC): SAS Institute.
- Locatello L, Rasotto MB, Evans JP, Pilastro A. 2006. Colourful male guppies produce faster and more viable sperm. *J Evol Biol.* 19:1595–1602.
- Lotem A, Wagner RH, Balshine ES. 1999. The overlooked signaling component of nonsignaling behavior. *Behav Ecol.* 10:209–212.
- Low M. 2006. The energetic cost of mate guarding is correlated with territorial intrusions in the New Zealand stitchbird. *Behav Ecol.* 17:270–276.
- Marshall TC, Slate J, Kruuk LEB, Pemberton JM. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol Ecol.* 7:639–655.
- McGraw KJ, Adkins-Regan E, Parker RS. 2005. Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. *Naturwissenschaften.* 92:375–380.
- Michl G, Török J, Griffith SC, Sheldon BC. 2002. Experimental analysis of sperm competition mechanisms in a wild bird population. *Proc Natl Acad Sci USA.* 99:5466–5470.
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfsen G. 2008. Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. *Mutat Res/Genet Toxicol Environ Mutagen.* 650:210–216.
- Møller AP, Surai PF, Mousseau TA. 2005. Antioxidants, radiation and mutation as revealed by sperm abnormality in barn swallows from Chernobyl. *Proc R Soc Lond B Biol Sci.* 272:247–252.
- Nilsson J-Å. 2002. Metabolic consequences of hard work. *Proc R Soc Lond B Biol Sci.* 269:1735–1739.
- Nowicki S, Searcy WA. 2004. Song function and the evolution of female preferences: why birds sing, why brains matter. *Ann NY Acad Sci.* 1016:704–723.
- Partali V, Liaaen-Jensen S, Slagsvold T, Lifjeld JT. Carotenoids in food chain studies II: The food chain of *Parus* spp. monitored by carotenoid analysis. *Comp Biochem Physiol Part B Biochem Mol Biol.* 87:885–888.
- Peters A, Denk AG, Delhey K, Kempenaers B. 2004. Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. *J Evol Biol.* 17:1111–1120.
- Petrie M, Williams A. 1993. Peahens lay more eggs for peacocks with larger trains. *Proc R Soc Lond B Biol Sci.* 251:127–131.
- Royle NJ, Surai PF, Hartley IR. 2001. Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? *Behav Ecol.* 12:381–385.
- Royle NJ, Surai PF, McCartney RJ, Speake BK. 1999. Parental investment and egg yolk lipid composition in gulls. *Funct Ecol.* 13:298–306.
- Safran RJ, Neuman CR, McGraw KJ, Lovette IJ. 2005. Dynamic paternity allocation as a function of male plumage color in barn swallows. *Science.* 309:2210–2212.
- Saladin V, Bonfils D, Binz T, Richner H. 2003. Isolation and characterization of 16 microsatellite loci in the Great Tit *Parus major*. *Mol Ecol Notes.* 3:520–522.
- SAS®. 2003. Release 9.1. Cary (NC): SAS Institute.
- Sheldon BC. 2000. Differential allocation: tests, mechanisms and implications. *Trends Ecol Evol.* 15:397–402.
- Slate J, Marshall T, Pemberton J. 2000. A retrospective assessment of the accuracy of the paternity inference program CERVUS. *Mol Ecol.* 9:801–808.
- Surai PF. 2002. Natural antioxidants in avian nutrition and reproduction. Nottingham (UK): Nottingham University Press.
- Surai PF, Blesbois E, Grasseau I, Chalah T, Brillard J-P, Wishart GJ, Cerolini S, Sparks NHC. 1998. Fatty acid composition, glutathione peroxidase and superoxide dismutase activity and total antioxidant activity of avian semen. *Comp Biochem Physiol Part B Biochem Mol Biol.* 120:527–533.
- Szentirmai I, Komdeur J, Székely T. 2005. What makes a nest-building male successful? Male behavior and female care in penduline tits. *Behav Ecol.* 16:994–1000.
- Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. 1998. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod.* 13:1429–1436.
- Velando A, Beamonte-Barrientos R, Torres R. 2006. Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment. *Oecologia.* 149:535–542.
- Vishwanath R, Shannon P. 1997. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reprod Fert Dev.* 9:321–331.
- von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc R Soc Lond B Biol Sci.* 266:1.
- Walker G. 1977. Role of male great tit in nest-building. *Br Birds.* 70:123.
- Ward S, Speakman JR, Slater PJB. 2003. The energy cost of song in the canary, *Serinus canaria*. *Anim Behav.* 66:893–902.
- Wiersma P, Selman C, Speakman J, Verhulst S. 2004. Birds sacrifice oxidative protection for reproduction. *Proc R Soc Lond B Biol Sci.* 271 (Suppl):S360–S363.
- Williamson KA, Surai PF, Graves JA. 2006. Yolk antioxidants and mate attractiveness in the Zebra Finch. *Funct Ecol.* 20:354–359.