

Adjustment of female reproductive investment according to male carotenoid-based ornamentation in a gallinaceous bird

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Abstract Carotenoid-based ornaments (many yellow–orange–red colourations) may signal the genetic or parental quality of the bearer. Thus, their expression could influence the amount of resources/energy that the mate will invest in the production of offspring, thereby optimising its reproductive fitness. The differential allocation hypothesis (DAH) predicts that females mated with more attractive males should lay more and better eggs. This has been explored only in few bird species with carotenoid-based traits. We tested this hypothesis in the red-legged partridge (*Alectoris rufa*), a gallinacean with very variable laying capacity. Both sexes display carotenoid-based ornamentation that gradually fades throughout the laying period. Here, the redness of beak and eye rings of captive males was intensified after mating by means of paint. The

proportion of females that laid eggs did not differ between treatments. Amongst laying females, those mated with colour-enhanced males (experimental females) tended to lay earlier and produced significantly more eggs than controls, but of similar quality (egg mass and composition). We additionally investigated whether male attractiveness influenced egg components depending on the clutch size and laying sequence. The testosterone level in eggs from experimental females was positively related to the laying order, whereas control eggs did not show any trend. Our results provided mixed support for the DAH, but nevertheless revealed that female red-legged partridges may adjust their breeding investment according to male carotenoid-based ornamentation.

Keywords Androgens · Mate choice · Maternal effects · Maternal hormones · Reproductive compensation · Sexual selection

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Introduction

In sexually reproducing and iteroparous species, an accurate assessment of mate quality is crucial to correctly estimate the costs and benefits of mate choice and is also the best resource allocation strategy during current and successive reproductive events (Edward and Chapman 2011; Stearns 1992). The perceived quality of the mate would determine whether choosers increase or decrease their post-mating reproductive investments (Burley 1986; Gowaty 2003). Sexual signals have evolved in this context, revealing individual quality and serving to optimize mate choice and reproductive decisions (Horvathova et al. 2012).

Amongst signals allowing to assess the quality of a potential mate, morphological traits, such as body size, the

size of certain body appendices, sexual exhibitions, elaborated songs and colourations, can be cited (Andersson 1994). Carotenoid-based colourful traits are amongst the commonest sexual ornaments in birds and other vertebrates, attracting considerable attention from evolutionary biologists (Hill 2006; McGraw 2006). Carotenoids are pigments not synthesized by animals that must be obtained from the diet and hence are usually considered as limited resources for signallers (Endler 1980; Hill 1990). Furthermore, carotenoids are necessary for maintaining the body homeostasis, acting as immunostimulant and antioxidant molecules (Lozano 1994; von Schantz et al. 1999; Pérez-Rodríguez 2009). Thus, it has been hypothesized that carotenoids constitute a currency in the physiological trade-off between reproduction (here sexual signalling) and self-maintenance (Lozano 1994; von Schantz et al. 1999; Alonso-Alvarez et al. 2008). Such a role ultimately implies that carotenoid-based ornaments are costly and only fully expressed by high-quality individuals, making them reliable signals of individual quality (i.e. handicap signals; Zahavi 1975).

The role of carotenoid-based signals in mate choice decisions has been demonstrated by correlational studies, as well as manipulations of the ornament expression (e.g. Houde 1997; Møller et al. 2000; Hill 2006). However, the same does not apply for post-mating reproductive decisions. Numerous studies amongst different taxa have demonstrated experimentally that individuals take resource allocation decisions on the basis of a variety of sexual signals of their mates (reviewed in Sheldon 2000; also recently, e.g. Kotiaho et al. 2003; Galeotti et al. 2006; Skinner and Watt 2007). Nevertheless, examples for carotenoid-based ornaments are limited to few bird species, including the zebra finch (*Taeniopygia guttata*: e.g. Burley 1986; Gil et al. 1999), two seabirds (*Sula nebouxii*: Velando et al. 2006; *Larus michahellis*: Morales et al. 2009), and more recently, the mallard (*Anas platyrhynchos*: Giraudeau et al. 2011).

The ability of individuals to adjust post-mating investment depending on the sexual signals of their mates includes many parental care traits (sensu Clutton-Brock 1991), from gamete formation to offspring independence (e.g. Sheldon 2000; Grootuis et al. 2005). In birds, females may not only allocate more or less resources to alter egg mass or clutch size (review in Horvathova et al. 2012) but may also modify the amount of certain substances into the egg. In fact, carotenoids are present not only in sexual signals but also in the egg yolk, protecting the embryo from oxidative stress associated with its high anabolic turnover (Surai 2002; Pérez-Rodríguez 2009). Female birds may differentially allocate carotenoids to eggs based on their mate attractiveness (i.e. Saino et al. 2002; Navara et al. 2006a; Szigeti et al. 2007; Bolund et al. 2009). For instance, female zebra finches allocated more carotenoids to first eggs in the clutch when mated with males carrying green rings, which are

perceived as less attractive (Williamson et al. 2006). Female birds may also adjust their reproductive investment through the differential allocation of hormones into eggs. The best known example is that of maternal androgens. It was initially suggested that larger amounts of androgens in the yolk enhance offspring competitiveness and development (i.e. Schwabl 1996), though recent reviews provide a mixed support to this original assertion, and emphasize the complexities of this mechanism (Grootuis et al. 2005; Carere and Balthazart 2007; Gil 2008; but see Cucco et al. 2008). In any case, most studies show an increase in the level of yolk androgens in those females mated with more ornamented males (Kingma et al. 2009 and references therein).

Here, we experimentally tested whether females of a gallinaceous precocial bird, the red-legged partridge (*Alectoris rufa*), modulate their reproductive investment depending on mate attractiveness. This species is socially monogamous (although mate switching and extra-pair paternity have been reported; Casas et al. 2006) and exhibits a peculiar double-nesting breeding system (Green 1984; Casas et al. 2009). Females often lay two simultaneous clutches in different nests: one is incubated by its social mate and the other by the female, each one being of ca. 11 eggs (average, but higher clutch size in nest incubated by males; Casas et al. 2009). Interestingly, about 40% of females (average; range 0–55%, depending on years) exhibit this double-nesting behaviour (Casas et al. 2009). Females laying a single-clutch incubate alone (males do not help with incubation but with nest guarding; Casas 2008). All this means that the investment that females put into producing eggs is particularly important and also very variable, making this bird species particularly well-suited for studying adaptive reproductive investment strategies according to male phenotype. Both male and female partridges display carotenoid-based red traits (beaks, eye rings and legs), with males being redder than females (Villafuerte and Negro 1998; Pérez-Rodríguez 2008). Redness peaks around the start of egg laying and progressively fades throughout the laying period (Pérez-Rodríguez 2008; Alonso-Alvarez et al. 2008). It is known that avian bare parts can brighten or fade within days or even hours (Rosen and Tarvin 2006; Velando et al. 2006; Gautier et al. 2008; Ardia et al. 2010) and may act as dynamic signals being continuously evaluated by females throughout reproduction (Velando et al. 2006). The same could occur in the case of red-legged partridges. Furthermore, we know that redder male red-legged partridges have better condition and immunocompetence, as well as fewer parasites and greater antioxidant capacity (Pérez-Rodríguez and Viñuela 2008; Pérez-Rodríguez et al. 2008, 2010; Alonso-Alvarez et al. 2008; Mougeot et al. 2009). We thus assume that redder male partridges should be high quality and probably preferred males. Here, captive female partridges were paired with control males or instead with males whose redness was artificially intensified. In contrast with zebra finch studies where red rings

were used as a proxy of bill colour, we directly manipulated the red bill and eye ring of partridges by using makeup and paint.

The differential allocation hypothesis (DAH; Burley 1986) proposes that choosers increase their reproductive investment when paired with an attractive mate because attractive mates would be of higher quality, investing more in parental care or transmitting better genes to descendants (Burley 1986; Sheldon 2000). We first tested specific predictions of the DAH, namely, that females mated with more attractive males (here, redder males) should be more likely to lay a clutch, to lay earlier, to have more eggs and to have eggs of better quality (greater carotenoid content). For this, we assessed female investment in reproduction within each group, measuring: (1) the latency to the first egg, (2) the total number of eggs produced, (3) egg/yolk mass, and (4) egg yolk levels of carotenoids.

Secondly, we further explored variations in female breeding investment by investigating how the egg composition (yolk carotenoids and androgens) varied with both the clutch size and laying sequence, depending on the male redness (experimental treatment). Egg quality often varies with the total number of eggs laid, and with laying order, so we can expect that some effects on egg quality could be revealed by interactions between the treatment and the total number of eggs or laying order (e.g. Kingma et al. 2009; Bortolotti et al. 2003). However, predicting the direction of such interactions is difficult on the basis of the current literature.

Methods

Experimental procedure

The study was carried out at the *Dehesa de Galiana* experimental facility (Instituto de Investigación en Recursos Cinegéticos, Ciudad Real, Spain). It was conducted on captive partridges provided by a governmental breeding facility (Chinchilla; Junta de Comunidades de Castilla La Mancha, JCCM) located in Albacete (Spain), which maintains a population exclusively obtained from wild birds captured in a nearby land (15,000 hectares) that belongs to the Spanish Army. We used 60 adult partridges forming 30 pairs that were kept in separate outdoor cages (i.e. 30 cages, one for each pair, of $1 \times 0.5 \times 0.4$ m) at ambient temperature and under natural photoperiod. Birds were fed ad libitum with commercial pelleted food containing xanthophylls (Superfeed, Spain) and wheat in a 1:1 proportion.

Each pair was assigned to one of two treatments (control pairs: $N=16$; experimental pairs: $N=14$). In order to avoid initial biases, the age of the birds was balanced between groups ($\chi^2=0.12$, $df=1$, $P=0.73$ in both sexes). While the 28 birds were older than 2 years (14 males and 14 females), the remaining 32 birds were 1-year-old individuals. Most pairs

consisted of birds of the same age, except for six pairs (three of them composed by a young male and an older female, and the other three with the opposite age composition). Body size (tarsus length; accuracy 0.01 mm), body mass (accuracy 1 g), and body condition (body mass over tarsus length residuals; males: $R^2=0.30$, $P=0.002$; females: $R^2=0.28$, $P=0.004$) were also balanced between both treatments [one-way analysis of variances (ANOVAs), all P values > 0.40]. This balanced sample would contribute to avoid age-related and state-mediated effects, as it has been suggested that age and condition may mask the pattern of differential allocation (Harris and Uller 2009).

Manipulation started on April 14, 2008, 1 week after pair formation. In our population, this date coincides with the period of highest levels of circulating carotenoid and red colour expression, prior to the laying period (Pérez-Rodríguez 2008). Therefore, we modified male colouration at the end of the mating season and throughout the laying period of our captive population.

Colour manipulation

In experimental pairs, we manipulated the redness of eye rings and beak in males only. For the eye rings, the red colour was intensified by carefully applying Superstay Lipcolour 510 (Maybelline, New York) with a paintbrush. In control pairs, males received a similar manipulation, but using a clean paintbrush. For the beak, the red colour of males from experimental pairs was manipulated using red nail paint (Yolizul Cosmetics ref. L0312754, Barcelona, Spain), whereas transparent nail paint was applied to the beak of males of the control pairs (the last contained the same components that red nail paint, excepting the red pigment; Yolizul Cosmetics ref. L0312740, Barcelona, Spain). The paint in the beak was dried with a hairdryer. All the manipulation required about 10–15 min/bird. Since colours were exposed to weather conditions (sunlight and rain) and abrasion (through contact with the cages), birds were reviewed and repainted weekly throughout the experiment.

Eye ring and beak colour were measured using a portable spectrophotometer (Minolta CM-2600 d; Tokyo, Japan). Reflectance spectrum was determined from 360 to 700 nm wavelength at 10-nm intervals. In order to estimate colour variability (i.e. redness), the hue of each trait was calculated from reflectance data by applying the segment classification method (Saks et al. 2003). Lower hue values, in degrees, are indicative of redder traits. Duplicated colour measurements of both eye ring and beak were taken before and after painting. These duplicates were repeatable (R^2 range = 0.75–0.94, all $P < 0.001$; following Lessells and Boag 1987, here and thereafter), and average values were used for analyses. At the start of the experiment, males from both treatments did not differ in their carotenoid-based ornaments

(one-way ANOVAs: $F_{1,28}=0.69$, $P=0.41$ and $F_{1,28}=1.23$, $P=0.28$ for eye ring and beak hue, respectively). Afterwards, as expected, coloured-males showed redder eye rings than controls (mean \pm SE: $42^\circ\pm 0.1$ vs. $63^\circ\pm 0.2$, for coloured and control males, respectively) and the same was found for beak redness ($30^\circ\pm 0.2$ vs. $42^\circ\pm 0.2$; all pairwise comparisons P values <0.1). The mean hue of coloured-male eye rings (42°) was close but not within the range of our sample before manipulation (43° – 93°), whereas mean beak hue (30°) was within such limits (28° – 58°).

Egg production and quality

Female egg production was monitored until the first week of July (end of the laying period). Cages were inspected daily to collect eggs ($N=188$) on the day they were laid. Eggs were individually marked with a pencil (date and female identity) and immediately stored at 16°C to stop embryo development (Thear 1987). Then they were weekly transferred to an ultralow freezer (-85°C), where they were kept in hermetic plastic boxes to avoid dehydration. In captivity, red-legged partridges, such as other gallinaceans, lay eggs without a clear clutch sequence, so we avoided using the term clutch size and instead used ‘number of eggs’.

Total egg mass (accuracy 0.001 g) was determined before embryo development. It was previously determined in other sample that total egg mass at this time highly correlates with egg volume ($r=0.92$, $P<0.001$, $N=780$). Since the volume in itself should have little consequence for embryo development we chose to use only egg mass in our analyses. Upon the day of biochemical analyses, eggs were carefully opened and the yolk was weighed (accuracy 0.001 g). Each yolk was divided in several aliquots, each one including all the concentric layers of the yolk (i.e. semi-hemispheres). Aliquots were used to determine carotenoid and androgen concentrations.

Carotenoid levels in the egg yolk

Carotenoids were isolated from the yolks following Surai and Speake (1998) with some modifications. Briefly, 0.5 g yolk were diluted in 0.5 ml of bidistilled water and repeatedly mixed in 2 ml ethanol plus 1 ml bidistilled water. Hexane (2.5 ml) was added, and the mixture vortexed during 10 s and afterwards centrifuged at $2026\times g$ during 5 min. The supernatant was removed to a tube and evaporated under nitrogen flow. Then, the extract was resuspended in 1 ml methanol, vortexed and filtered (nylon, $0.2\ \mu\text{m}$) and the absorbance of the new fluid assessed by spectrophotometry (Shimadzu UV-1603, Japan) at 446 nm in duplicate ($R^2=0.90$, $p<0.001$; e.g. Pérez-Rodríguez 2008; Alonso-Alvarez et al. 2009). The final concentration was determined by using a standard curve of lutein (Sigma Chemicals). Mean values of duplicate

measurements were used in the statistical analyses. Total carotenoid amounts in the egg yolk were also calculated by multiplying carotenoid concentration per total yolk mass. High total carotenoid amounts may imply a prolonged exposure to these substances during development, whereas carotenoid concentration may affect certain phases of embryonic development (Safran et al. 2008).

A validation test was performed on a random subsample of 40 egg yolks that were simultaneously analyzed by high-performance liquid chromatography (HPLC) and spectrophotometry. HPLC analyses were performed by Agilent 1100 Series using a Spherisorb type ODS2, $5\ \mu\text{m}$ C18, reverse-phase column, $4.0\ \text{mm}\times 250\ \text{mm}$ (Waters Spherisorb) with a mobile phase was bidistilled water (A) and methanol (B). Samples were eluted with 10% of A and 90% of B, during 2 min, changing with a 5 min gradient to 100% of B and then were maintained during 18 min. Finally, system returned to initial conditions in 5 min, using a flow rate of 0.8 ml/min, and detection by absorbance at 475 nm. The total carotenoid concentration calculated from all the carotenoid peaks in HPLC was very highly correlated with the concentrations calculated from spectrophotometer absorbances ($R^2=0.99$, $p<0.001$, $N=40$). Hence, we assume that carotenoid levels in our statistical models accurately represent total carotenoid levels in the yolk.

Androgen levels in the egg yolk

Two of the main androgens found in the avian egg yolk are androstenedione and testosterone, the first being more abundant than testosterone in some species (e.g. precocial birds; Groothuis et al. 2005) also showing effects on offspring development (e.g. Hegyi et al. 2011). Hence, we quantified both androgens in the egg yolk of red-legged partridges by analyzing the odd eggs in the laying sequence. In this order, 50 mg of yolk samples were homogenized in 1 ml of bidistilled water and vigorously vortexed. Androgens were extracted by adding 3 ml of a mixture of petroleum and diethyl ether (30:70) to the sample, vortexing for 15 min and centrifuging for 10 min (4°C , $1400\times g$). The ether phase was decanted after snap-freezing the tube in an alcohol bath at -70°C . This procedure was repeated a second time, and both ether phases were combined in a single tube and evaporated under stream of nitrogen to dryness. The dried extract was redissolved in 0.5 ml of ethanol 90%, kept at -20°C overnight and centrifuged for 10 min (4°C , $700\times g$) to remove proteins. Supernatants were dried under a stream of nitrogen, and the dried extract was redissolved in steroid-free serum (DRG International, Mountainside, NJ). Androstenedione and testosterone concentrations in the extracts were determined using enzyme immunoassay kits also from DRG International. The same protocol has been successfully used to quantify avian yolk steroids in several bird species,

showing high recovery rates (>90 %; López-Rull and Gil 2009a, b). Sensitivity for testosterone and androstenedione is established at 0.083 ml and 0.019 ng/ml, respectively. Repeatabilities (i.e. Lessells and Boag 1987) calculated from a subset of samples ($n=30$) measured twice were high (testosterone: $r=0.95$; androstenedione: $r=0.90$).

Statistical analyses

We used the SAS software (v8.2, SAS Institute 2001), and the GENMOD procedure to test for differences between treatments in laying probability (with a binomial error) and in the number of eggs produced throughout the experiment by those females that laid (with a Poisson error). To correct for overdispersion in the Poisson distribution, the PSCALE option was used (SAS Institute 2001). Differences in the latency until the onset of egg laying were also analysed using GENMOD, but since this variable met the normality criterion, normal error was used (here, nonlaying couples were excluded). The influence of the treatment tested as a fixed factor in both GENMOD models.

Variability in egg composition variables (i.e. egg mass, yolk mass, proportion of yolk mass regard to egg mass, and yolk carotenoid, androstenedione and testosterone concentrations and total amounts) was explored by means of Generalized Mixed models (PROC MIXED in SAS; Littell et al. 2006). Dependent variables or their residuals meet the normality assumption. The influence of the treatment was tested as a fixed factor. The position of each egg with regard to others (i.e. laying order) and the total number of eggs produced throughout reproduction were tested as covariates. In order to facilitate the interpretation of results, both covariates were centred, following Schielzeth (2010). The interactions between the treatment and both covariates were also tested. When testing for covariates or treatment \times covariate interactions, both the slope (covariate) and the intercept

(identity of the pair) were always included as random factors, following Schielzeth and Forstmeier (2009). Degrees of freedom were adjusted by using the Satterthwaite approximation. Least square means, SE as well as estimated slopes and their SE were calculated from these models. Additionally, the effect size of the main comparison (colour-enhanced vs. control pairs) was calculated from least square means and SD of the models (Table 1). The odds ratio (OR) was also calculated for the binomial test (laying probability).

To avoid overfitting (Forstmeier and Schielzeth 2011), initial models did not include parents' age and their interactions. However, as previously indicated (above), these factors were homogeneously distributed between treatments. The Akaike information criterion (AIC) was used to select the best fitted model. We report saturated full models including the two covariates, the treatment and the two first order interactions in the electronic supporting material (ESM). In text, we only report results from best fitted final models.

Results

Treatment effects on laying probability and clutch size

Four females from control pairs and six from experimental pairs did not lay any egg. Overall, the proportion of females that laid did not differ between treatment groups ($\chi^2=1.05$, $df=1$, $P=0.305$; OR=0.44). We further analyzed treatment effects on egg production and quality, considering only those pairs in which the female produced a clutch (i.e. excluding nonlayers).

Amongst females that laid (i.e. 12 control and 8 experimental pairs), those paired with colour-enhanced males laid almost two times more eggs than females paired with control males (Fig. 1a), this difference between treatment

Table 1 Comparisons of the reproductive output of female captive partridges paired with control or colour-enhanced males ($n=12$ and 8, respectively)

Dependent variable	Control		Colour-enhanced		Effect size
	Mean	SD	Mean	SD	
Number of eggs	1.872	0.924	2.716	0.606	1.08
Latency to first egg (day)	3.374	0.794	2.565	0.430	1.26
Egg mass (g)	17.757	1.372	18.097	1.178	0.27
Yolk mass (g)	6.231	0.767	6.400	0.683	0.23
Yolk proportion (%)	35.229	2.655	35.384	2.302	0.06
Carotenoid concentration ($\mu\text{g/g}$)	10.459	3.786	8.351	2.844	0.63
Carotenoid amount (g)	67.111	26.773	53.598	20.196	0.57
Androstenedione concentration (ng/g)	11.020	2.832	11.329	2.502	0.12
Androstenedione amount (ng)	68.825	20.985	73.343	18.937	0.23
Testosterone concentration (ng/g)	2.856	1.346	2.913	1.134	0.05
Testosterone amount (ng)	18.289	9.163	19.413	7.709	0.13

Cohen's d was used to estimate the effect size

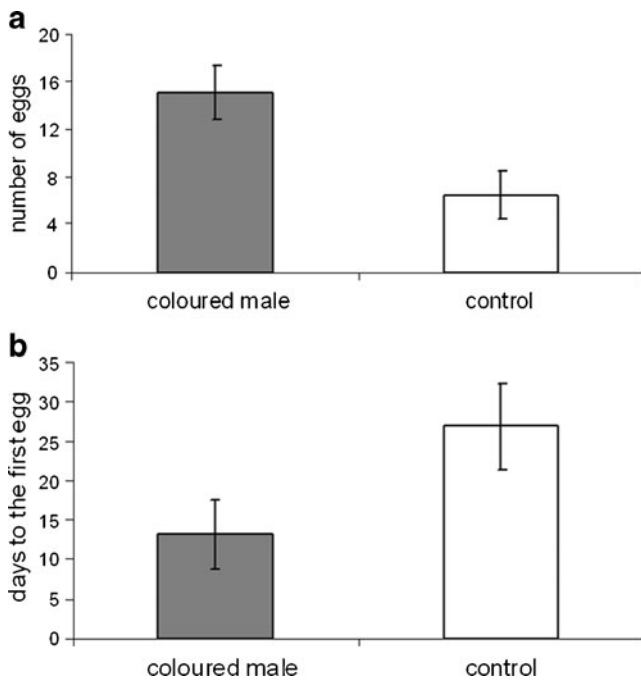


Fig. 1 Means±SE (a) number of eggs produced by laying female red-legged partridges (b) number of days elapsed between treatment and first egg lay date according to treatment (filled bars: females mated with a colour-enhanced male; empty bars: females mated with a control male)

groups being significant ($\chi^2=6.29$, $df=1$, $P=0.012$). Moreover, females paired with colour-enhanced males laid earlier (lower latency to first egg) than females paired with control males, although this difference was not significant (Fig. 1b; $\chi^2=3.30$, $df=1$, $P=0.069$).

Treatment effects on egg quality: egg mass, yolk mass and yolk carotenoid content

Egg mass variability was only explained by laying order, although the relationship was not significant ($F_{1,6.75}=3.70$, $P=0.097$). Similarly, only laying order explained variability in egg yolk mass ($F_{1,4.12}=8.14$, $P=0.045$). Both variables were negatively related to laying order (slopes±SE: $-0.033\pm$

0.018 and -0.022 ± 0.008 , respectively). The treatment did not affect egg mass ($F_{1,17.3}=0.35$, $P=0.560$), yolk mass ($F_{1,17.3}=0.35$, $P=0.609$) or the proportion of yolk/egg mass ($F_{1,17.3}=0.35$, $P=0.891$).

Variation in yolk carotenoid levels (concentration and absolute values) was only explained by the number of eggs laid in interaction with the experimental treatment (Table 2). In control females, which produced fewer eggs overall, carotenoid concentration in the egg yolk decreased with total egg production ($F_{1,3.65}=5.23$, $P=0.026$; slope±SE: -0.402 ± 0.176), whereas in females paired with colour-enhanced males, which produced more eggs, this relationship was not significant ($F_{1,6.82}=0.05$, $P=0.827$; slope±SE: $+0.025\pm 0.112$; see Table 2 and Fig. 2). The interaction showed a trend to significance when testing the variability in the absolute amount of carotenoids per egg (treatment×number of eggs interaction: $F_{1,6.04}=5.79$, $P=0.053$; slopes±SE: -2.445 ± 1.251 and $+0.682\pm 0.692$, for control- and colour-enhanced male mates, respectively). In this model, laying order was negatively related to carotenoid amounts ($F_{1,3.61}=16.5$, $P=0.019$; slope±SE: -1.338 ± 0.329). The treatment did not show a significant influence on carotenoid yolk concentration ($F_{1,6.82}=2.25$, $P=0.178$) or total carotenoid amounts in yolk ($F_{1,7.8}=1.86$, $P=0.211$).

Treatment effects on yolk androgen content

Variability in androstenedione yolk concentration was not explained by any term, including the interaction between laying order and treatment (Table 3 and Fig. 3a). Nonetheless, eggs laid by females mated with colour-enhanced males showed a significant increase in androstenedione levels with laying order ($F_{1,3.08}=20.93$, $P=0.019$; slope±SE= $+0.333\pm 0.073$; Fig. 3a), whereas this relationship was not found for eggs laid by females from control pairs ($F_{1,5.1}=0.12$, $P=0.742$; slope±SE= $+0.081\pm 0.233$). The same interaction was not significant in the model testing total androstenedione amount in the egg ($F_{1,4.85}=2.25$, $P=0.196$). Similarly, eggs laid by experimental females showed a significant increase in androstenedione amounts ($F_{1,6.56}=$

Table 2 Best fitted mixed model testing the influence of several factors and covariates on the variability of carotenoid concentration in the egg yolk (see details in ‘Methods’)

	Estimate	SE	F values	df	P values
Colour treatment	0.739	1.181	0.39	1,4.48	0.562
Number of eggs	0.029	0.128	4.48	1,6.75	0.074
Colour treatment×number of eggs (estimate for control group)	-0.423	0.172	6.03	1,6.75	0.045
Random factors	Estimate	SE	Z values	P values	
Bird identity	3.417	2.837	1.20	0.114	
Residual	10.363	1.235	8.39	<0.001	

See also ESM and the model for total carotenoid amounts in eggs (i.e. not concentration) given in ‘Results’

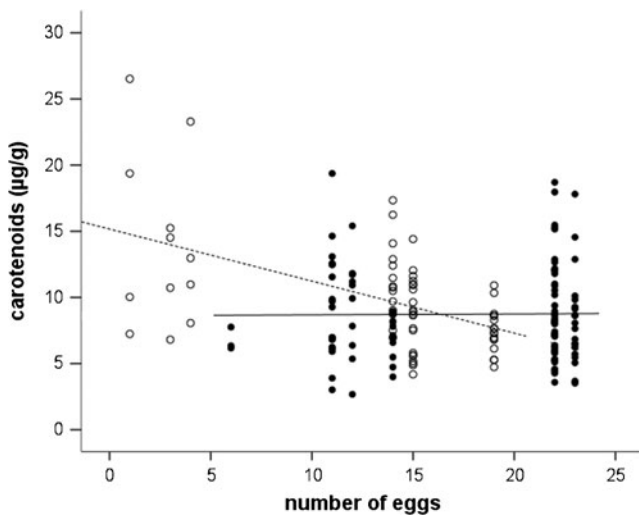


Fig. 2 Relationship between carotenoid concentration in an egg produced by a female red-legged partridge and the number of eggs produced by the same female. *Dotted line and empty circles* represent data from female mated with control males, whereas *continuous line and filled circles* show data from females mated with a colour-enhanced male. *Values and regression lines* were obtained from raw data. See slopes from the mixed models in ‘Results’. Note that the number of eggs covariate was, however, centred in the model (see ‘Statistical analyses’)

11.84, $P=0.012$; slope \pm SE: $+1.980\pm 0.468$), whereas eggs from control females did not ($F_{1,4.99}=0.10$, $P=0.760$; slope \pm SE: $+0.493\pm 1.527$). The effect of treatment on androstenedione concentrations or amounts was not significant ($F_{1,9.95}=0.06$, $P=0.819$ and $F_{1,10.8}=0.21$, $P=0.657$, respectively).

In the case of testosterone concentration (Table 4), females mated with colour-enhanced males allocated more testosterone to later eggs ($F_{1,3.67}=10.16$, $P=0.038$; slope \pm SE: $+0.154\pm 0.048$; Fig. 3b). In control females, testosterone levels and laying order were unrelated ($F_{1,3.27}=1.69$, $P=0.278$; slope \pm SE: -0.132 ± 0.101 ; Fig. 3). Similarly, the same treatment \times laying order interaction was significant when considering the total testosterone amount in the yolk ($F_{1,8.28}=8.78$, $P=0.017$). Experimental females showed a positive relationship ($F_{1,4.31}=9.26$, $P=0.035$; slope \pm SE:

$+1.001\pm 0.329$), whereas controls did not show the link ($F_{1,4.67}=1.18$, $P=0.329$; slope \pm SE: -0.863 ± 0.793). Similarly to androstenedione, the effect of treatment on testosterone concentrations or amounts was not significant ($F_{1,10.2}=0.01$, $P=0.928$ and $F_{1,9.87}=0.04$, $P=0.842$, respectively; see also Table 1).

Discussion

Despite of a limited sample size (number of pairs), our experimental results show that female red-legged partridges are able to differentially invest in their progeny depending on the level of carotenoid-based ornamentation of their mate during the laying period. However, our experiment only provided mixed support for the DAH (see below). Those females paired with redder males were not more likely to lay, but when they did, they produced more eggs and tended to lay earlier than controls. When considering egg quality, we did not find support for that females mated with redder males increased egg mass, yolk mass or overall carotenoid allocation to eggs, as predicted by the DAH.

Nevertheless, the results as a whole support the idea that carotenoid-based ornaments are not only involved in mate choice but also influence post-mating resource allocation decisions, although some of these adjustments were subtle and depended on the laying sequence. The only examples of the cited adjustments come from several experiments on few bird species. The best known model is the zebra finch. Male colouration in this species is manipulated by means of rings recreating the beak colour of males. Females mated with males carrying red rings biased offspring sex ratio to males (i.e. the most expensive sex in terms of parental investment; Burley 1986; see Trivers 1972) and also allocated more androgens to the egg yolk (Gil et al. 1999). Rutstein et al. (2004) showed that female zebra finches mated with red-ringed males produced heavier eggs compared to controls, but the effect was only found in one of two breeding rounds and in interaction with female body mass. Gilbert et al. (2006) also showed that female

Table 3 Mixed model testing the influence of several factors and covariates on the variability of androstenedione concentration in the egg yolk (see ‘Methods’ and ‘Results’)

	Estimate	SE	F values	Df	P values
Colour treatment	-0.607	1.368	0.20	1,9.61	0.667
Laying order	0.306	0.119	3.41	1,6.57	0.110
Colour treatment \times laying order (estimate for control group)	-0.251	0.196	1.64	1,6.57	0.243
Random factor	Estimate	SE	Z values	P values	
Bird identity	5.08	3.302	1.54	0.062	
Bird identity \times laying order	0.059	0.069	0.85	0.197	
Residual	5.512	1.107	4.98	<0.001	

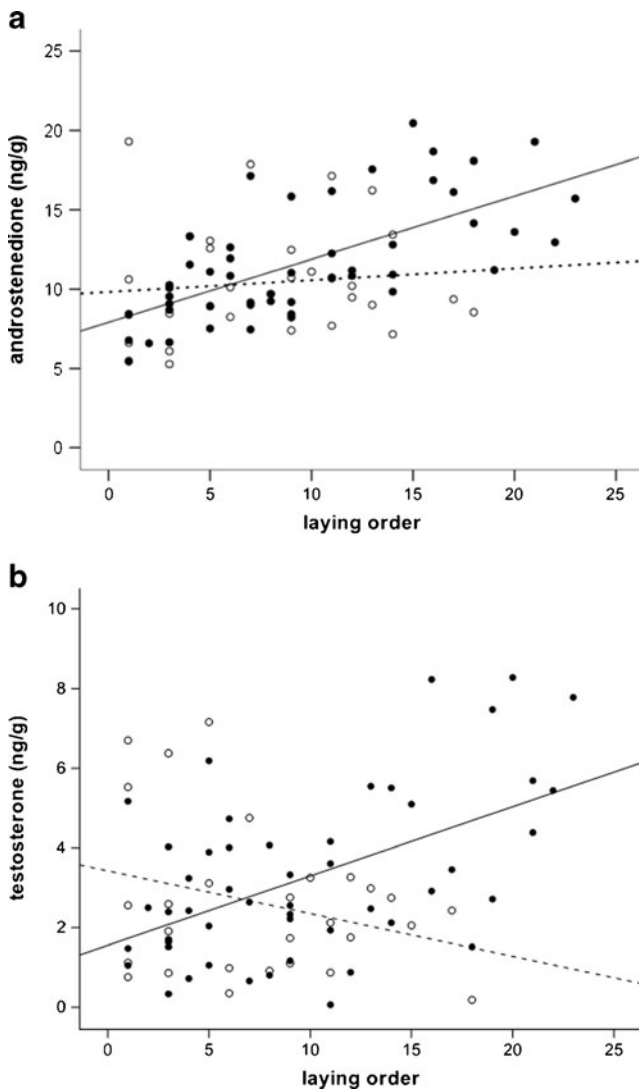


Fig. 3 Relationship between **a** androstenedione and **b** testosterone concentrations in the egg yolk and laying order. *Dotted line and empty circles* represent data from females mated with control males, whereas *continuous line and filled circles* show data from females mated with a colour-enhanced male. *Values and regression lines* were obtained from raw data. See slopes from the mixed models in ‘Results’

zebra finches mated with red-ringed males produced heavier eggs at the end of the laying sequence. They also showed that females mated with less attractive (green-ringed) males produced female offspring with shorter tarsus. Finally, Williamson et al. (2006) showed that females mated with green-ringed males allocated larger amounts of carotenoids and vitamin E to the first eggs of the laying sequence, but lower levels at the end. We must nonetheless indicate that part of these studies did not consider the random slopes when passing from testing main experimental effects to treatment \times covariate (e.g. laying order) interactions, which could have led to type I errors (see Schielzeth and Forstmeier 2009 and Forstmeier and Schielzeth 2011).

Studies manipulating individual attractiveness by directly modifying the ornament colouration are limited to two sea-bird species. The feet of male blue-footed boobies (*S. neobuxii*) was made duller by means of makeup, which induced females to reduce the size of the last (second) egg of the clutch (Velando et al. 2006; Dentressangle et al. 2008). In yellow-legged gulls (*Larus michaellis*), the enlargement of the red surface of the bill in one of the pair members induced higher feeding rates to chicks by its mate (Morales et al. 2009).

In contrast with direct colour manipulations, Giraudeau et al. (2011) have recently intensified the colour of the bill of male mallards by increasing carotenoid levels in their diet, which led females to lay larger eggs and also eggs with larger antibacterial content (i.e. lysozyme). Although the manipulation of carotenoid availability may somehow constitute a more natural approach, we must consider that carotenoids simultaneously affect different body functions (Lozano 1994; von Schantz et al. 1999) that could, in turn, alter individual behaviour (see Helfenstein et al. 2008). In other words, by manipulating male carotenoid ornaments via the diet, it is difficult to know what part of the female investment was only due to ornament expression.

In the present study, female red-legged partridges paired with red-painted males were not more likely to lay, but when they did, they produced more eggs and showed differential allocation of carotenoids and testosterone to eggs. The

Table 4 Best fitted mixed model testing the influence of several factors and covariates on the variability of testosterone concentration in the egg yolk (see ‘Methods’)

	Estimate	SE	F values	df	P values
Colour treatment	−0.498	0.606	0.67	1,9.77	0.431
Laying order	0.151	0.050	0.02	1,9.14	0.900
Colour treatment \times laying order (estimate for control group)	−0.290	0.090	10.39	1,9.14	0.010
Random factor	Estimate	SE	Z values	P values	
Bird identity	0.703	0.616	1.14	0.127	
Bird identity \times laying order	0.004	0.011	0.42	0.336	
Residual	2.647	0.515	5.14	<0.001	

allocation of these substances, however, depended on the total number of eggs produced and on the laying order, respectively (Figs. 2 and 3). The carotenoid content of eggs produced by control females showed a significant negative correlation with the number of eggs laid, whereas eggs from females mated with colour-enhanced males did not show such a relationship. Here, the effect, however, seems mostly to be due to those control females laying very few eggs, which allocated larger carotenoid amounts to eggs (Fig. 2). We must anyway be cautious as females mated with coloured males did not produce very small clutches (Fig. 2). The result, nonetheless, suggests that control females managed a fixed carotenoid budget, which was distributed amongst more or less eggs. In agreement with this, Bortolotti et al. (2003) showed that the number of eggs produced by a female red-legged partridge negatively correlates with its circulating carotenoid level at the end of laying, and Pérez-Rodríguez (2008) showed that females lose their redness and decrease circulating carotenoid levels at higher rates than males throughout the laying period.

With regard to androgens, species-specific increases and decreases in yolk androgen levels with laying order have been reported in a variety of bird species, though increases are more frequent (reviews in Groothuis et al. 2005; Gil 2008). Similarly, within-species variability has also been reported (Groothuis et al. 2005; Gil 2008), but as far as we know, only Navara et al. (2006b) have previously reported an interactive effect of mate attractiveness and laying order upon yolk androgen levels. In that study conducted on house finches (*Carpodacus mexicanus*), Navara et al. (2006b) did not manipulate colour, but found an opposite pattern: females allocated more androgens to later eggs when mated with paler males, whereas females mated with redder males did not show differences. We have instead found an increase in testosterone levels throughout the laying sequence in females mated with colour-enhanced males, but not in control pairs. Increases in androgen values across the laying sequence could function to mitigate the disadvantage of later-hatched chicks (see Müller et al. 2004; Groothuis et al. 2005). Female red-legged partridges mated with redder mates, when producing more eggs, could favour the survival of later hatchlings by increasing androgen allocation to eggs. In support of this, grey partridge (*Perdix perdix*) chicks whose yolk testosterone levels were artificially increased by injections of the hormone into the egg developed a better immunocompetence and grew faster than controls (Cucco et al. 2008).

Most studies manipulating carotenoid-based ornaments and testing its impact on post-mating parental decisions (above) apparently support the DAH proposed by Nancy Burley (1986). She suggested that choosers increase their reproductive investment when paired with an attractive mate because attractive mates would also be high-quality

individuals that will invest more in parental care (Burley 1986) or transmits better genes to descendants (Sheldon 2000), therefore increasing the fitness returns of the investment. However, our experiment provided only mixed support for DAH. We did not find that female mated with redder males were more likely to lay, although we found that when they did, they tended to lay earlier and produced significantly more eggs. Moreover, we had little evidence that treatment alone influenced egg yolk carotenoid content, and therefore that females mated with redder males increased egg quality. In any case, this could be related with the double-nesting behaviour of the red-legged partridge (Casas et al. 2009) where females mated with redder males could lay enough eggs to complete two different clutches.

Attractiveness may not necessarily predict greater offspring viability (Gowaty 2008). In this line, the compensatory hypothesis (Gowaty 2003; 2008) proposes that choosers may alternatively invest more when mated with a nonpreferred mate. This is only expected in mating systems where the chooser is highly constrained in its mate choice decision and where nonpreferred mating reduces the proportion of descendants able to breed (i.e. it reduces offspring viability; Gowaty et al. 2007). Under these conditions, the compensatory hypothesis predicts that individuals should try to do the ‘best-of-a-bad-job’ by increasing fecundity (offspring number) and hence by increasing the total number of descendants that survive and contribute to future generations (Gowaty 2008; see also Harris and Uller 2009 and Ratikainen and Kokko 2010). In the red-legged partridge, both mate switching during the same season and extra-pair mating have been documented (Casas et al. 2006; 2008), suggesting that partridges are not constrained in mate choice and therefore might be better candidates for a differential allocation strategy (i.e. Burley 1986). Our finding that, amongst laying female partridges, those mated with redder males produced more eggs (a fecundity proxy) apparently contradicts the compensatory investment (Gowaty 2008). But females could compensate not for the lack of mate quality, but for collateral effects of being paired with an ‘excessively attractive’ individual. If so, the allocation pattern of partridges could be the consequence of a conflict between parents over care (Houston et al. 2005; Hinde and Kilner 2007). It has been demonstrated that the expression of carotenoid-based traits in male red-legged partridges is favoured by high blood testosterone levels (Alonso-Alvarez et al. 2009), which negatively interferes with parental care, including incubation behaviour, as shown in other bird species (e.g. Ketterson and Nolan 1999; Alonso-Alvarez 2001). Sexiest and highly testosteroneized males would thus invest more in mating with additional females (Ketterson and Nolan 1999; Magrath and Komdeur 2003). Females mating with redder males could obtain indirect benefits as their offspring will be sexier and hence obtain more grand-

offspring. In this case, females paired with the most attractive males should try to offset the potential lack of paternal care by increasing egg numbers and/or offspring quality (e.g. accelerating chick growth). We must keep in mind that in the wild female red-legged partridges lay one or two clutches (in separate nests). When only one clutch is laid, it is incubated by the female (Cramp and Simmons 1980), but when two clutches are produced, the male incubates the second one without female help (Green 1984; Casas et al. 2009). Moreover, we have recently found that, in the wild, if a female partridge is paired with a redder male, she is most likely to lay more eggs in two clutches, one that she will incubate and the other one that its male will incubate (Mougeot et al., unpublished data). Houston et al. (2005) argued that a female that handicaps herself by strategically laying large clutches is unable to care alone and hence forces the male to care (also Smith and Härdling 2000). Similarly, other authors suggested that high yolk androgen levels may serve to counteract the lack of paternal care of attractive mates by improving offspring competitive skills (Groothuis et al. 2005; Moreno-Rueda 2007; Müller et al. 2007). Thus, the allocation patterns here described may also agree with a compensatory strategy (i.e. Gowaty 2003, 2008), but with greater reproductive investment in females paired with very attractive and perhaps also nonpreferred mates (see Ratikainen and Kokko 2010). In any case, if redder males are initially noncollaborating mates that become incubating birds or instead simply better parents is unknown. Both alternatives are difficult to disentangle.

In summary, although our results only provided mixed support for the DAH hypothesis, they overall showed that female red-legged partridges are able to adjust their reproductive investment depending on the carotenoid-dependent ornamentation levels of their social mate. Significant effects were detected despite the relatively small sample size. In order to better understand subtle breeding adjustments, further studies on larger samples should now be performed, preferably in the wild. Both redness and plasma carotenoid levels of male red-legged partridges gradually decline throughout the laying and postlaying periods (Alonso-Alvarez et al. 2008), so we suggest that these traits may be used by females to perform fine-tuned adjustments in their breeding investment, as recently showed in other avian species (Velando et al. 2006; Dentressangle et al. 2008; Morales et al. 2009).

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ELECTRONIC SUPPORTING MATERIAL

Full models testing variability egg composition:

Table S1. Saturated model testing variability in carotenoid concentration in the yolk.

	Estimate	SE	<i>F</i>	df	<i>P</i>
Colour treatment	0.524	1.117	0.22	1,5.27	0.658
Laying order	-0.175	0.123	2.39	1,2.05	0.260
Number of eggs	0.129	0.128	0.99	1,12.8	0.339
Colour treatment x laying order (estimate for control group)	0.031	0.206	0.02	1,2.05	0.894
Colour treatment x number of eggs (estimate for control group)	-0.443	0.187	5.62	1,12.8	0.034
Random factors	Estimate	SE	<i>Z</i>		<i>P</i>
Bird identity	2.87	2.371	1.21		0.113
Bird identity x laying order	0.065	0.109	0.60		0.274
Residual	9.175	1.221	7.51		<0.001

Table S2. Saturated model testing variability in total carotenoid amounts in the yolk.

	Estimate	SE	<i>F</i>	df	<i>P</i>
Colour treatment	3.703	8.784	0.18	1,4.49	0.693
Laying order	-1.396	0.419	10.11	1,5.12	0.024
Number of eggs	1.445	0.962	0.03	1,7.77	0.870
Colour treatment x laying order (estimate for control group)	0.222	0.808	0.08	1,5.12	0.794
Colour treatment x number of eggs (estimate for control group)	-3.113	1.313	5.62	1,7.77	0.046
Random factors	Estimate	SE	<i>Z</i>		<i>P</i>
Bird Identity	200.75	156.52	1.28		0.099
Bird identity x laying order	0.152	0.744	0.20		0.419
Residual	398.60	49.97	7.98		<0.001

Table S3. Saturated model testing variability in androstenodione concentration in the yolk.

	Estimate	SE	<i>F</i>	df	<i>P</i>
Colour treatment	-0.719	0.961	0.24	1,4.88	0.645
Laying order	0.280	0.129	1.91	1,4.49	0.231
Number of eggs	0.132	0.159	0.54	1,12.1	0.477
Colour treatment x laying order (estimate for control group)	-0.243	0.229	1.12	1,4.49	0.344
Colour treatment x number of eggs (estimate for control group)	-0.088	0.240	0.13	1,12.1	0.722
Random factors	Estimate	SE	<i>Z</i>		<i>P</i>
Bird identity	5.210	4.234	1.23		0.109
Bird identity x laying order	0.071	0.088	0.80		0.212
Residual	5.511	1.107	4.98		<0.001

Table S4. Saturated model testing variability in androstenodione amounts in the yolk.

	Estimate	SE	<i>F</i>	df	<i>P</i>
Colour treatment	-7.689	10.613	0.52	1,5.27	0.450
Laying order	1.612	0.759	1.08	1,3.96	0.358
Number of eggs	1.334	1.146	1.33	1,11.3	0.272
Colour treatment x laying order (estimate for control group)	-1.760	1.409	1.56	1,3.96	0.281
Colour treatment x number of eggs (estimate for control group)	-0.707	1.697	1.33	1,11.3	0.272
Random factors	Estimate	SE	<i>Z</i>		<i>P</i>
Bird identity	276.06	213.13	1.30		0.098
Bird identity x laying order	1.802	3.28	0.55		0.291
Residual	297.64	60.85	4.89		<0.001

Table S5. Saturated model testing variability in testosterone concentration in the yolk.

	Estimate	SE	<i>F</i>	df	<i>P</i>
Colour treatment	-0.484	0.674	0.52	1,6.58	0.497
Laying order	0.145	0.053	0.01	1,7.59	0.942
Number of eggs	0.018	0.076	0.10	1,16.3	0.759
Colour treatment x laying order (estimate for control group)	-0.298	0.106	7.96	1,7.59	0.024
Colour treatment x number of eggs (estimate for control group)	0.001	0.117	0.001	1,16.3	0.993
Random factors	Estimate	SE	<i>Z</i>		<i>P</i>
Bird identity	0.872	0.774	1.13		0.130
Bird identity x laying order	0.005	0.012	0.40		0.344
Residual	2.660	0.526	5.06		<0.001

Table S6. Saturated model testing variability in testosterone amounts in the yolk.

	Estimate	SE	<i>F</i>	df	<i>P</i>
Colour treatment	-3.504	4.484	0.61	1,5.36	0.468
Laying order	0.858	0.404	0.07	1,6	0.804
Number of eggs	0.254	0.509	0.30	1,14.3	0.593
Colour treatment x laying order (estimate for control group)	-1.914	0.763	6.29	1,6	0.046
Colour treatment x number of eggs (estimate for control group)	-0.077	0.789	0.01	1,14.3	0.924
Random factors	Estimate	SE	<i>Z</i>		<i>P</i>
Bird identity	37.49	38.31	0.98		0.164
Bird identity x laying order	0.384	0.754	0.51		0.305
Residual	119.47	24.32	4.91		<0.001