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Phenotypic variation in nestlings of a bird of prey under contrasting breeding and diet conditions

AUDREY STERNALSKI[1,2]*, FRANÇOIS MOUGEOT[3] and VINCENT BRETAGNOLLE[2]

[1]*Instituto de Investigación en Recursos Cinegéticos (IREC-CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain*
[2]*CEBC-CNRS, 79360 Beauvoir-sur-Niort, France*
[3]*Estación Experimental de Zonas Áridas (EEZA-CSIC), Carretera de Sacramento s/n, 04120 La Cañada de San Urbano, Almería, Spain*

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Environmental conditions often vary in space and time, and this may explain variation in the expression of phenotypic traits related to individual quality, such as ornamental coloration. Furthermore, the direction and strength of the relationship between coloured trait expression and individual quality might vary under contrasting conditions. These issues have been explored in adult birds but much less so in nestlings, which are more likely to experience different selective pressures and different physiological trade-offs than adults. Here, we empirically investigated the effects of contrasting breeding and diet conditions on the expression of carotenoid-based colour traits displayed by marsh harrier (*Circus aeruginosus*) nestlings. We studied the variation in coloration, body condition, and immune responsiveness of nestlings in four populations over a 5-year period. We characterized spatiotemporal differences in rearing conditions experienced by *C. aeruginosus* nestlings in terms of breeding (laying date, clutch size, and number of nestlings hatched and fledged) and diet (percentage of mammal in diet and prey diversity) conditions. We found that breeding conditions influenced the co-variation between coloration and immune responsiveness in female nestlings, and that diet conditions influenced the condition-dependence of nestling coloration in later-hatched nestlings. In addition, breeding conditions influenced nestling body condition and immune responsiveness, whereas diet conditions influenced nestling coloration and body condition. Our study highlights that nestling phenotype (levels of signalling, circulating carotenoids, and immunity) varies both spatially and temporally, and that some of this variation is related to differences in breeding and diet conditions. Moreover, under contrasting conditions, the direction of the relationships between nestling carotenoid-based coloration and nestling quality may also vary. In order to fully understand the evolution and maintenance of colour traits in nestling birds, studies and experiments should ideally be replicated under contrasting rearing conditions. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, ••, ••–••.

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INTRODUCTION

Knowledge of how conspicuous, brightly coloured traits evolved and are maintained as honest signals of individual quality remains critical to understanding sexual selection and animal communication theories. Both ideas are based on the assumption that signals are costly to express and that the cost of displaying conspicuous, brightly coloured traits varies with individual quality: only high-quality individuals (those in prime condition) should be able to afford this cost, thereby ensuring reliable signalling (Zahavi, 1975; Grafen, 1990). Although this theory is widely accepted (Andersson, 1994), empirical studies supporting the basic evidence that conspicuous trait expression positively correlates with measures of individual quality still generate conflicting results, with almost any relationship between these two parameters being found in well-studied species (e.g. Bolund, Schielzeth

*Corresponding author. E-mail: audrey.sternalski@gmail.com

& Forstmeier, 2009). One explanation for the existence of such discrepancy resides in environmental heterogeneity. Environmental conditions may enhance or constrain the relative differences between individuals of low and high quality (David *et al.*, 2000; Cotton, Fowler & Pomiankowski, 2004b; Cornwallis & Uller, 2009), and thereby affect the strength and/or the direction of the relationship between conspicuous trait expression and measures of individual quality (Candolin, 2000; Fargallo *et al.*, 2007; Vergara *et al.*, 2011, 2012).

Among animals, and especially birds, many yellow–red conspicuous traits displayed by adults are coloured by carotenoid pigments (Hill & McGraw, 2006). Carotenoid pigments are not synthesizable by vertebrates, and must be acquired through their diet (Goodwin, 1984). Besides their function in signalling, carotenoids also serve various alternative physiological functions. For instance, they can act as enhancers of immunity or as scavengers of the free radicals produced in intense metabolic processes like rapid growth, immune responses, or stressful conditions (von Schantz *et al.*, 1999; Møller *et al.*, 2000; Surai, 2002; Pérez-Rodríguez *et al.*, 2008). Hence, individuals may have to balance the use of these pigments to colour conspicuous ornaments against these other physiological functions, and the resulting trade-offs could ensure the reliable signalling of quality (Lozano, 1994). As the availability of carotenoid to the individual depends on the ingestion of carotenoid precursors from the environment, and upon the physiological status of individuals, the resolution of such trade-offs, and therefore the relationships between colour-trait expression and measures of individual quality, may vary in relation to diet and environmental conditions (Hill, 1999; Martínez-Padilla *et al.*, 2010).

Environmental heterogeneity is known to influence the expression of sexual ornaments displayed by adult birds (Hill, Inouye & Montgomerie, 2002) and their relationships with measures of individual quality (Candolin, 2000; Dunn *et al.*, 2010; Vergara *et al.*, 2012). However, much less attention has been paid to such possible effects in nestlings, despite the latter sometimes displaying traits that are carotenoid-based (Kilner, 2006). The evolution of carotenoid-based traits in nestlings probably results from different environmental selective pressures than in adults, because sexual selection is not acting on immature individuals. A signalling function in parent–offspring communication has been proposed to explain the evolution of such colour traits in nestlings, with coloration informing parents about an offspring's need (Kilner, 1997), quality, or value (Saino *et al.*, 2000, 2003; Loiseau *et al.*, 2008; Dugas & McGraw, 2011). Nestlings also differ from adults in their physiological status (e.g. rapid growth rate or an immune system that is still developing), and may experience different physiological trade-offs than adults, for instance a trade-off between growth and immunity (Soler *et al.*, 2003; Brommer, 2004). Despite these differences, environmental heterogeneity might also influence the average expression of carotenoid-based traits in nestlings and their co-variation with measures of quality. Previous studies have demonstrated that both natural food variation and manipulation of diet early in life influenced the expression of conspicuous coloration in nestlings (Fitze, Tschirren & Richner, 2003; Fargallo *et al.*, 2007; Sternalski *et al.*, 2010). However, these studies have largely neglected potential spatial and temporal variation effects, as they have been conducted in single populations or over short time periods. We propose that a broader examination, which takes into account the differences in breeding and diet conditions in which the nestlings are reared, as a surrogate of environmental heterogeneity, will offer new insights.

In the present study, we used marsh harrier (*Circus aeruginosus*) nestlings within size-structured families to empirically examine the influence of contrasting breeding and diet conditions on the expression of carotenoid-based traits of wild nestlings. *Circus aeruginosus* is a sexually dimorphic raptor (i.e. size dimorphism is already apparent at the nestling stage, with females being the larger sex; Riedstra, Dijkstra & Daan, 1998) characterized by hatching asynchrony (Bavoux, Burneleau & Bretagnolle, 2006). This creates a marked body mass and size difference between nestlings in relation to their sex and hatching order, with first-hatched and female nestlings being heavier and larger than later-hatched and male nestlings. These differences in mass and size strongly influence the levels of sibling competition (Magrath, 1990; Mock & Parker, 1997). *Circus aeruginosus* nestlings develop yellow carotenoid-pigmented bare parts (cere and tarsi) as early as 1 week old, similar to adults and other harrier species (e.g. Sternalski *et al.*, 2010, Sternalski *et al.*, 2012). In adults, these traits may function in mate choice (e.g. Montagu's harrier *Circus pygargus*; Mougeot & Arroyo, 2006), whereas in nestlings, a function in parent–offspring communication is likely. Experimental manipulation of carotenoid-based coloration in nestlings of this species influences mass gain and provisioning by parents, and increases brood reduction in male nestlings (A. Sternalski, unpubl. data), thereby supporting the idea that food provisioning by parents is adjusted to changes in nestling coloration, as is found more generally in other birds (e.g. Ewen *et al.*, 2008; Griggio, Morosinotto & Pilastro, 2009), including some raptors (e.g. Parejo *et al.*, 2010; J. M. Aviles, D. Parejo, unpubl. data).

Over a 5-year period, we studied *C. aeruginosus* nestlings from four populations that strongly differed in terms of habitat and diet. We investigated whether contrasting breeding and diet conditions influenced the average expression levels of nestling carotenoid-based coloration, body condition, and immune responsiveness (as a response to a phytohaemaglutinin, PHA, challenge). When breeding conditions are good, food may be less limited, and brood sizes are typically larger (Newton, 1998). Hence, nestlings should be in better body condition, should invest more in carotenoid-based signalling, and should have better immune responsiveness. In populations and years when the diet is dominated by mammal prey, which are energy-rich but carotenoid-poor, nestlings might be in better body condition but more carotenoid limited, and therefore may display paler carotenoid-based coloration, or might display carotenoid-based coloration to the detriment of immune responsiveness, which is sometimes boosted by greater carotenoid availability (Blount *et al*., 2003; McGraw & Ardia, 2003). We further expected the influence of breeding and diet conditions to differ between nestlings according to their competitive ability within a brood. Both energy and carotenoid acquisition depend on sibling competition, so the effect of environmental conditions may be more pronounced for poorer competitors (i.e. males or later-hatched nestlings in *C. aeruginosus*). We also expected contrasting local environmental conditions to affect the strength and/or the direction of the relationship between colour-trait expression and nestling body condition and immune responsiveness. Because rearing conditions may enhance or constrain the relative differences between low- and high-quality individuals, we expected stronger relationships (e.g. greater slopes) to be found under more adverse rearing conditions (Fargallo *et al*., 2007; Vergara *et al*., 2011, 2012).

MATERIAL AND METHODS

Study sites and prey availability

We studied *C. aeruginosus* nestlings during five consecutive breeding seasons (2006–2010) in four study sites located in central western France (Charente–Maritime district): the Marais de Brouage (MB, ~72 km^2, 45°51′N, 1°04′W; see Bavoux *et al*., 1989 for more details), the Marais de Rochefort (MR, ~30 km^2, 46°04′N, 0°98′W; see Butet & Leroux, 2001 for more details), the Ile de Ré (IR, ~36 km^2, 46°20′N, 1°43′W), and the Marais Poitevin (MP, ~65 km^2, 46°28′N, 1°08′W). MB and MR are wetland areas. MB mainly consists of grassland habitats with many small reed beds. MR is characterized by more intensive agriculture (Butet & Leroux, 2001), with scarce and very small reed beds, in highly fragmented patches. In contrast, IR and MP are dominated by arable land. IR is a large coastal island where arable crops are mainly potatoes and vineyards; it is now managed for salt production, and consists of a mixture of low hills created for salt extraction, grassland and wetland habitats, and woodlands. MP is a typical intensive farmland mainly used for cultivating winter cereals and oilseed rape, spring-sown crops, and with few pastures and other permanent or semi-permanent crops used for livestock grazing.

The *C. aeruginosus* is a generalist predator that has a diverse diet, consisting of birds, fishes, reptiles, insects, and small- and large-mammals (Bavoux *et al*., 1990; Clarke, 1995). However, diet is highly variable among populations and years (Sternalski *et al*., in press), and strongly differed between the four sites, with decreasing diet diversity (Shannon index) from MP, MR, IR, to MB (Sternalski *et al*., in press). Overall, *C. aeruginosus* consumed mostly mammals and some fish in MP, but mostly fish in MB and MR. In IR, the diet of *C. aeruginosus* was restricted to mammals and game birds (Sternalski *et al*., in press). In addition, when a particular prey species is abundant, such as the common vole (*Microtus arvalis*) that shows cyclic variations in abundance (Salamolard *et al*., 2000; see also Ingenbleek *et al*., 2004), *C. aeruginosus*s may specialize on this prey to the detriment of other prey species. This was the case in two populations (MP and MR), where the frequency of small mammals in the diet might reach more than ~40% during years of peak vole abundance (Sternalski *et al*., in press). Therefore, in years when *M. arvalis* are at their population peaks, these dominate the diet of *C. aeruginosus* in MP and MR. Conversely, when voles are scarce, the diet of *C. aeruginosus* is more diverse. The four study sites thus strongly differed both in terms of habitat types and in terms of food availability and prey types.

Breeding performance and phenotypic variation in nestlings

Harrier nests were located during the pre-laying period (March–April) within each study site. Not all sites were monitored in all years, but in total 15 different site-years were monitored (see Table S1 for sampling details). Nests were visited as soon as possible to assess reproduction stage, and were checked again roughly four times during the breeding period to record clutch size (i.e. number of laid eggs per clutch), hatched brood size (i.e. number of hatched eggs per clutch), and fledged brood size (i.e. number of fledged young per brood). Laying date (i.e. laying date of the first egg laid) was estimated from direct

observation (some nests were visited during egg laying, providing exact laying dates) or by backdating from the hatching date, which was itself estimated from egg density or from chick age upon first visit after hatching (see Millon, Arroyo & Bretagnolle, 2008; Sternalski *et al.*, 2010). For the latter case, we assumed 33 days of incubation (Simmons, 2000).

At the first nest visit soon after hatching, each chick was head-marked using a non-toxic marker pen to allow identification within a brood, and was ranked according to hatching order. When older, nestlings were individually ringed and wing-tagged. Hatching order (hereafter referred to as rank) was categorized using two classes (first-hatched chick and later-hatched, younger, chicks), following Arroyo, De Cornulier & Bretagnolle (2002). When chicks were close to fledging (upon the last nest visit, at nestling age: 31 ± 4 days), we measured body mass (with a Pesola scale, to the nearest 1 g), wing length (with a ruler, to the nearest 1 mm), and tarsus length (with a calliper, to the nearest 0.1 mm) to estimate body condition. We measured coloration of bare parts (with a colorimetric chart, see below), took a blood sample from the brachial vein using heparinized capillaries, and measured immune responsiveness to a PHA challenge (see below). Blood was kept refrigerated (0–5 °C) and centrifuged at 10 000 *g* within 4 h of collection. Plasma samples were stored at –20 °C until analysed. Pellets were used to genetically sex nestlings, following Fridolfsson & Ellegren, (1999).

Carotenoid-based coloration

Cere and tarsi coloration were measured by direct comparison with a yellow–orange colorimetric chart (Roche Yolk Colour Fan; Neuilly-sur-Seine, France) under shaded light conditions, a method previously used and validated (by comparing colour scores and colour measurements made using a spectrophotometer) for *C. pygargus* nestlings (see Sternalski *et al.*, 2010; Sternalski, Mougeot & Bretagnolle, 2012a). Cere and tarsi colour scores ranged from 0 (very pale yellow) to 6 (bright yellow), were highly repeatable ($r = 0.96$ and 0.94 for cere and tarsi, respectively), and were strongly and positively correlated (mixed model with year, site, and nest as a random effects: $F_{1,153} = 1993.66$, $P < 0.001$, $N = 326$; slope ± SE = 0.796 ± 0.056). As we were interested in the overall carotenoid-based coloration of nestlings (and the overall level of pigments used), we summed cere and tarsi scores to obtain a total nestling coloration score indicative of overall carotenoid-based coloration (see Dawson & Bortolotti, 2006; Sternalski *et al.*, 2010).

Circulating carotenoids

The carotenoid concentration in plasma was determined using a spectrophotometer. Plasma samples were diluted in acetone (1 : 6 dilution) and the mixture was vortexed and centrifuged at 10 000 *g* for 5 min to precipitate the flocculent proteins. The optical density of the supernatant was examined at 450 nm using microtiter plates and a Biotek Powerwave XS2 spectrophotometer (Winooski, Vermont, USA). Plasma carotenoid concentrations (μg mL^{-1}) were calculated using a lutein standard curve (Extrasynthese, ref. 0306 S), as lutein is the main pigment circulated in nestling *C. aeruginosus* (Sternalski *et al.*, 2012; see also Sternalski, Mougeot & Bretagnolle, 2012b). Repeatabilities within and between plates, estimated from a random subset of samples measured twice, were high (intraplate, $F_{14,15} = 26.3$, $P < 0.001$, $r = 0.92$; interplate, $F_{54,55} = 10.3$, $P < 0.001$, $r = 0.83$).

Assessment of immune responsivness

We measured the response to a PHA challenge, a technique routinely used in birds as an estimation of cellular immune responsiveness (Smits, Bortolotti & Tella, 1999). The PHA skin test measures some aspects of cellular immunity and pro-inflammatory potential in nestlings. It consists of an intradermal injection of PHA, which produces a prominent perivascular accumulation of T-lymphocytes followed by macrophage infiltration (Goto *et al.*, 1978). It produces a small but measurable swelling, the magnitude of which indicates aspects of an individual's ability to mount a cell-mediated immune response. Each nestling was injected with 0.5 mg of PHA (SIGMA L-8754) suspended in 0.1 mL of phosphate buffer solution (PBS) at a marked site on one wing web. We measured web thickness at the injection site with a pressure-sensitive dial thickness gauge (Teclock SI-112) to the nearest 0.01 mm. Web thickness was measured three times prior to the injection and three times again 24 h after the injection. Both the initial ($r = 0.93$) and the final measurements ($r = 0.97$) of wing web thickness were highly repeatable. We calculated PHA responses as the change at 24 h in average thickness (in mm) at the injection site.

Spatiotemporal variation of breeding and diet conditions

We first characterized the diet of *C. aeruginosus* within each study site and year from March to July using pellets, remains, and visual observations (see Sternalski *et al.*, in press). The combination of information from these three sources may improve raptor

diet analyses (Simmons, Avery & Avery, 1991), although some biases may still remain (Redpath *et al*., 2001). Pellets and remains were collected in nests during nest visits. We analysed differences in the diet composition (i.e. both number of prey items and biomass) between the four study sites, combining all sources of data. Overall, 281 pellets ($N = 61$ nests), 618 remains ($N = 156$), and 183 visual observations ($N = 91$) were obtained, representing 1171 identified prey items.

To characterize breeding and diet conditions, we first calculated the average (i.e. mean) value of the following six parameters for each study site and year: (1) laying date; (2) clutch size; (3) hatched brood size; (4) fledged brood size; (5) percentage of mammals in diet (i.e. percentage of small and large mammal prey items in diet); and (6) diet diversity index (Shannon index, $H' = -\Sigma\pi \log\pi$, where π represents the proportion of each species in the sample; Shannon & Weaver, 1949). In order to summarize the contrasting rearing conditions that the nestlings experienced, we conducted a principal component analysis (PCA) on these six mean population parameter values per site and year after scaling them. Our aim was not to tease apart the effects of specific environmental factors, knowing that these are often interrelated, but to summarize heterogeneity in rearing conditions with independent axes. In raptors, several breeding parameters such as laying date or clutch size are simultaneously influenced by environmental heterogeneity and by parental quality, but are typically much more influenced by the environment, and in particular by food abundance (Newton, 1979). Here, we did not try to disentangle the relative importance of environmental conditions and parental quality in explaining the variation in nestling phenotype. Because we studied sites that were characterized by contrasting habitats and diets, and because food abundance or prey type sometimes fluctuated greatly among study years, we suspect that most of the observed variation resulted from the environment (see Sternalski *et al*., in press). For instance, we found that laying date was earlier in MB than in the three other sites, clutch size increased from MB and MR to MP and IR, and fledged brood size was higher in IR and MP than in MB and MR (Sternalski *et al*., in press). Instead, in this study, we investigated the extent of this variation in contrasting rearing conditions (i.e. populations and years).

The PCA summarized the six population year parameters into two PC axes that explained 71% of variation. The first PC axis (hereafter EC1) was mostly influenced by average nest breeding parameters, with clutch size and hatched and fledged brood sizes having the highest positive loadings (with eigenvectors, EVs, of 0.53, 0.60, and 0.48, respectively), and with laying date having a negative loading in EC1 (EV –0.29). The first axis was mostly indicative of poor (low EC1 values: late breeding, small clutches and small fledged brood size) versus good (high EC1 values: earlier breeding, larger clutches and larger fledged brood sizes) breeding conditions. This association, commonly found in avian species such as raptors (e.g. Newton, 1998; Salamolard *et al*., 2000), characterizes breeding conditions, which usually depend on parental quality and environmental conditions like food abundance and availability (e.g. Wiehn & Korpimäki, 1997). We did not measure food abundance here, but EC1 was probably influenced by this factor, and is indicative of breeding investment and performance in each population and year. Breeding conditions, in turn, might affect within-brood competition levels. Under good breeding conditions, brood sizes are larger, and therefore brood competition levels between nestlings are also higher.

The second PC axis (hereafter EC2) was mostly influenced by diet composition, with the percentage of mammals in the diet having a high positive loading (EV 0.64), and with diet diversity index having a negative loading (EV –0.57). This second axis therefore differentiates between populations and years of different diets, independently of EC1 (i.e. breeding conditions). High EC2 values were indicative of populations in which the percentage of mammals in the diet was high, and in which the diet diversity was low. In contrast, negative EC2 values were indicative of a more diverse diet, with fewer mammals consumed. Small and large mammals are energy-rich but carotenoid-poor prey, unlike other prey items such as fishes, birds, reptiles, or insects (Goodwin, 1984). In situations where diet composition is mainly restricted to mammals (i.e. high EC2 values), nestlings could thus be more carotenoid limited, and consequently less coloured, whereas in situations where diet is diverse but few mammals are consumed (i.e. negative EC2 values), nestlings could be less carotenoid limited but also more energy constrained, and therefore in poorer condition (see Sternalski *et al*., 2010).

The first axis (EC1) explained 40% of variation (eigenvalue 2.40) and the second axis (EC2) explained a further 31% of variation (eigenvalue 1.84). Both EC1 and EC2 varied among sites and among years within a given site, and summarized the overall environmental heterogeneity across space and time (see Fig. S1).

Statistical analyses

We used general linear mixed models (mixed procedure, SAS 9.2) to test the effects of environmental conditions, nestling sex, nestling rank (first hatched versus later hatched), and their interactions on col-

oration, circulating carotenoid levels, body condition, and response to PHA challenge. All models included the variable 'age' as a fixed effect and the variable 'nest' as a random effect to take into account the non-independence of nestlings from the same brood. This random effect was always significant, and was therefore maintained in the models. However, when analyses were conducted on first-hatched nestlings only (one per brood), we used General Linear Models (GLM procedure; SAS, 2001). When investigating variation in body condition we used body mass (log-transformed) as the dependent variable, with age, age^2 (quadratic relationship with age), and log-transformed wing and tarsus lengths (as measures of nestling size) being included as explanatory variables. The residual body mass from this model was used as a measure of a nestling's body condition (hereafter referred to as body mass index).

For each nestling phenotypic trait (coloration, carotenoid levels, body mass index, and response to PHA), we first used initial explanatory models that included sex, rank, EC1, and EC2, and their interactions. We also tried models that included the quadratic terms EC1^2 and EC2^2, as well as all of their interactions with sex and rank, to test for possible nonlinear (quadratic) relationships between environmental conditions and phenotypic traits. Non-significant ($P > 0.05$) terms were sequentially removed from the initial models, starting with interactions, following a backward stepwise procedure, until only the significant explanatory variables or interactions were retained in the models. When significant interaction between fixed factors occurred, the statistical significance of each factor at different levels was computed using the LSMEANS statement (SAS, 2001).

We tested whether the relationship (slope) between body mass index and coloration or between response to PHA and coloration differed according to environmental condition (EC), by specifically testing whether the interactions coloration × EC1 or coloration × EC2 explained variations in the variable of interest, i.e. body mass index or response to PHA. We also tested for possible nonlinear (quadratic) effects of EC by including EC1^2 and EC2^2, and their interactions, with coloration as explanatory variables in our initial models. For these analyses, we only considered populations with a minimum sample size of five nestlings (see Table S1).

RESULTS

Rearing conditions and nestling phenotype

We first investigated whether EC1 and EC2 explained variation in average nestling coloration, circulating carotenoid levels, body mass index, and response to PHA challenge, and whether these effects differed according to nestling sex and rank (see Table S2 for the complete statistical results).

Variation in nestling coloration was explained by age, sex, and by EC2, depending on nestling rank, but was not explained by EC1 or any other interaction between these variables (see Table 1). The best relationship between coloration and EC2 was not linear but quadratic (significant EC2^2 and EC2^2 × rank interaction; Table 1). Coloration increased linearly with nestling age (Table 1), and nestling males were more coloured than were females (Least Square Means (LSMs) of 3.56 ± 0.15 and 3.19 ± 0.15, respectively). In addition, the average coloration varied non-linearly (quadratic relationship) with EC2. Coloration scores were lowest for intermediate EC2 values, and were higher for both the lowest and highest EC2 values, particularly in first-hatched nestlings (Fig. 1A). Overall, nestling coloration was therefore higher when the diet was either dominated by mammals or of low diversity, or when it was diverse with fewer mammals, but not when it was intermediate. In addition, first-hatched nestlings appeared to be more coloured than later-hatched nestlings when the diet was dominated by mammals and was of low diversity (highest EC2 values; Fig. 1A).

The variation in the circulating carotenoid levels of nestlings was explained by sex (mixed model, $F_{1,128} = 6.14$, $P = 0.014$), but was not explained by age, rank, EC1, EC2 (all $P > 0.10$), or any interaction between these variables (all $P > 0.15$). As for coloration score, carotenoid levels were higher in male than in female nestlings (LSMs of 6.998 ± 0.342 and 6.036 ± 0.345 μg mL^{-1}, respectively).

Variation in the average nestling body mass index was explained by sex, EC1 depending on rank, and EC2 (see Table 1). The body mass index was greater for female than for male nestlings (LSMs of 0.058 ± 0.008 and –0.057 ± 0.008, respectively), and varied nonlinearly with EC1 (significant EC1^2; Table 1), with a different relationship for first- and later-hatched nestlings (significant EC1 × rank interaction; Fig. 2A; Table 1). Body mass index also increased linearly with EC2, with heavier nestlings in periods when the diet was dominated by mammals (Fig. 1B; Table 1). In first-hatched nestlings body mass index significantly and linearly decreased with EC1 (slope ± SE: –0.016 ± 0.007; Fig. 2A). In later-hatched nestlings, body mass index varied nonlinearly with EC1, with higher body mass index for intermediate EC1 values, and lower body mass index for populations with either low or high EC1 values (Fig. 2A).

Variation in response to PHA challenge was explained by rank depending on sex, and by EC1, also depending on rank (see Fig. 2B; Table 2). In females, first-hatched nestlings mounted lower immune responses to PHA than later-hatched nestlings (LSMs

Table 1. Effects of environmental conditions (EC1 and EC2), nestling sex, and rank (first hatched versus later hatched) on the carotenoid-based coloration, body mass index, and response to phytohaemaglutinin (PHA) challenge in *Circus aeruginosus* nestlings. Initial (full) models included all interactions between EC1 and EC2 with sex and rank (see Supporting information). We tested for nonlinear relationship by also including the quadratic terms $EC1^2$ and $EC2^2$, as well as all their interactions with sex and rank. The general linear mixed models included the variable 'age' as a fixed effect and the variable 'nest' as a random effect

Dependent variable	Explanatory	*df*	*F*	*P*	Estimate ± SE
Coloration score					
	Age	1,160	25.11	< 0.001	0.095 ± 0.019
	Sex	1,160	7.34	0.007	(♀) –0.369 ± 0.136
	Rank (R)	1,160	3.37	0.068	(1st) –0.314 ± 0.171
	EC2	1,160	0.80	0.372	–0.238 ± 0.128
	$EC2^2$	1,160	4.83	0.029	0.071 ± 0.095
	EC2 × R	1,160	6.01	0.015	(1st) 0.270 ± 0.110
	$EC2^2$ × R	1,160	8.22	0.005	(1st) 0.236 ± 0.082
Body mass index					
	Sex	1,155	112.09	< 0.001	(♀) 0.115 ± 0.011
	Rank (R)	1,155	0.00	0.998	(1st) 0.000 ± 0.011
	EC1	1,155	2.65	0.105	0.000 ± 0.006
	$EC1^2$	1,155	7.02	0.009	–0.005 ± 0.002
	EC2	1,155	13.65	< 0.001	0.023 ± 0.006
	EC1 × R	1,155	7.81	0.006	(1st) –0.019 ± 0.007
Response to PHA					
	Sex (S)	1,87	0.13	0.724	(♀) 13.652 ± 9.219
	Rank (R)	1,87	3.98	0.049	(1st) 1.170 ± 9.875
	$EC1^2$	1,87	70.04	< 0.001	5.717 ± 1.033
	S × R	1,87	5.05	0.027	(♀, 1st) –32.365 ± 14.397
	$EC1^2$ × R	1,87	11.63	0.001	(1st) 6.366 ± 1.867

of 87.6 ± 7.7 and 103.9 ± 7.2 mm, respectively). In contrast, in males, first-hatched nestlings mounted greater responses to PHA than later-hatched nestlings (LSMs of 106.3 ± 8.1 and 90.3 ± 6.8 mm, respectively). In addition, responses to PHA varied nonlinearly with EC1, with lower responses for intermediate EC1 values, and greater responses for higher and lower EC1 values (Fig. 2B). This nonlinear relationship also depended on nestling rank, the quadratic relationship being more pronounced for first-hatched nestlings (Fig. 2B; Table 2).

Rearing conditions and the co-variation between body mass index and carotenoid-based coloration

We first investigated whether the co-variation between nestling coloration and body mass index varied under contrasting environmental conditions (testing whether variation in body mass index was explained by EC × coloration interactions), and whether these effects differed in relation to nestling sex and rank. The relationship between body mass index and coloration did not vary with EC1, but depended on EC2 and rank (coloration × EC2 × rank interaction; see Table 2). In first-hatched nestlings, the relationship between body mass index and coloration did not vary with EC2 (slope ± SE, 0.01 ± 0.005; Fig. 3). In contrast, in later-hatched nestlings, this relationship significantly varied with EC2 (slope ± SE, –0.012 ± 0.005; Fig. 3), with the slope of the body mass index–coloration relationship becoming negative for positive values of EC2, indicative of a diet dominated by mammals and being of low diversity (see Fig. 3).

Rearing conditions and the co-variation between immune responsiveness and carotenoid-based coloration

The relationship between response to PHA and coloration also varied with environmental conditions (Table 2). In this case it varied with EC1, but depended on nestling sex (significant coloration × EC1 × sex interaction; Table 2). In males, the slope of the relationship between response to PHA and coloration did not significantly vary with EC1 (non-significant coloration × EC1 interaction; Fig. 4). However, in female nestlings, the slope of the PHA response–coloration relationship varied with EC1

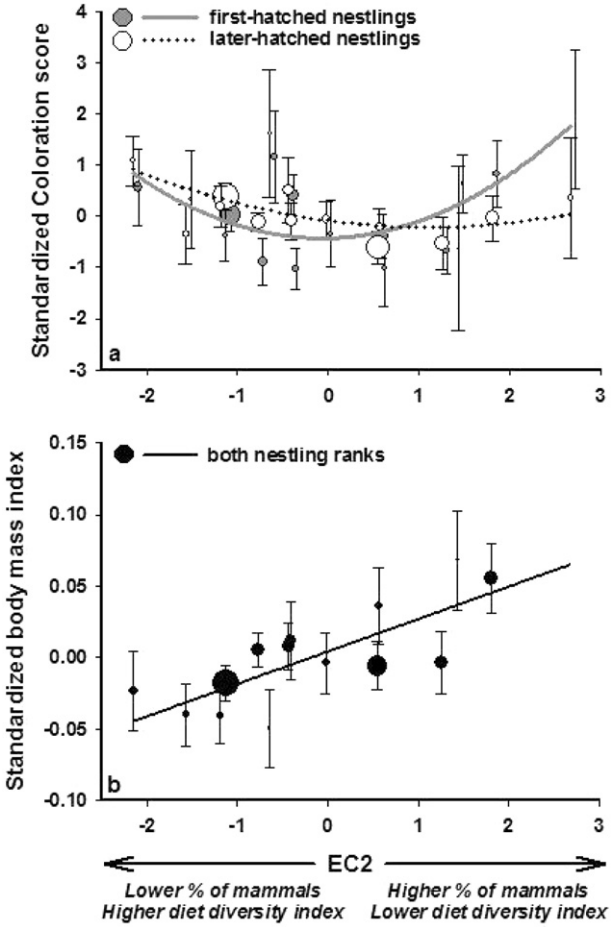

Figure 1. Effects of EC2 (i.e. 'diet conditions') on mean (±SE) nestling phenotype. A, standardized coloration score (i.e. coloration score corrected for nestling age and nestling sex), with nestling rank (first-hatched nestlings, dark-grey symbols; later-hatched nestlings, open symbols). B, standardized body mass index (i.e. body mass index corrected for nestling sex and EC1). Symbol sizes are indicative of sample sizes within each population/year (number of nestlings). Lines represent fitted linear or quadratic regressions for first-hatched (solid dark-grey line, A) and later-hatched nestlings (dotted line, A), and for all populations (solid black line, B).

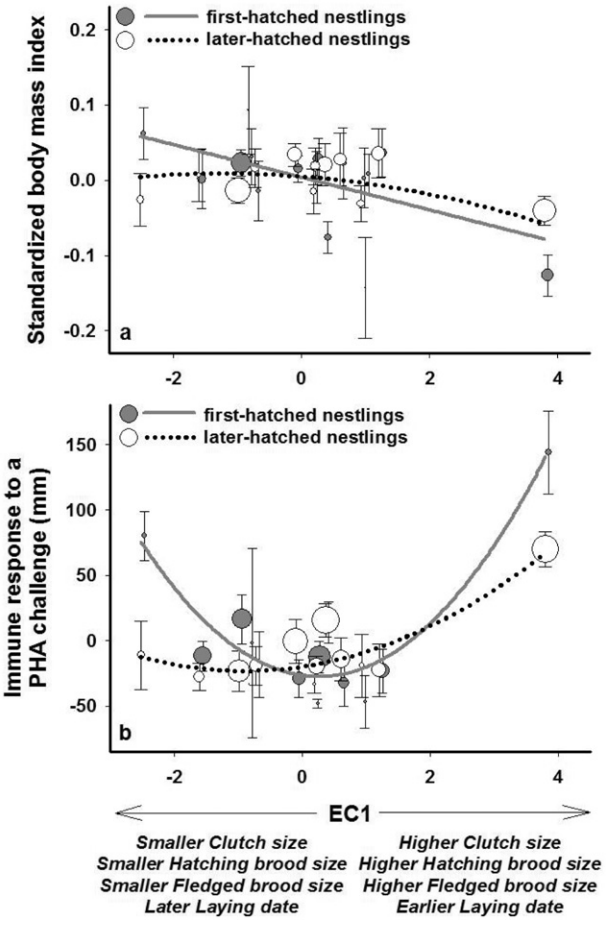

Figure 2. Effects of EC1 (i.e. 'breeding conditions') on mean (±SE) nestling phenotype. A, standardized body mass index (i.e. body mass index corrected for nestling sex and EC2). B, immune response to phytohaemaglutinin (PHA) challenge (mm). Symbol colours indicate nestling rank (first-hatched nestlings, dark-grey symbols; later-hatched nestlings, open symbols); symbols sizes are indicative of sample sizes within each population/year (number of nestlings). Lines represent fitted linear or quadratic regressions for first-hatched (solid, dark-grey line) and later-hatched nestlings (dotted line).

(significant coloration × EC1 interaction), and decreased with increasing EC1, indicative of higher breeding investment and performance (Fig. 4).

DISCUSSION

Rearing conditions and average nestling phenotype

Nestling coloration varied with diet conditions (EC2) but not with breeding conditions (EC1). The relationship with EC2 was complex (nonlinear, quadratic relationship), and depended on nestling rank. When diet was more diverse (lowest EC2 values), nestlings were more coloured, but coloration decreased when the percentage of mammals in the diet increased, under intermediate EC2 values. However, when the diet consisted mainly of mammals (highest EC2 values), nestling coloration also increased, particularly so in first-hatched nestlings. Carotenoid contents and levels differ between prey types (Goodwin, 1984). Mammals are rich in energy but poor in carotenoids, whereas preys such as reptiles, insects, or fishes are poorer in energy but richer in carotenoids (Goodwin, 1984). Therefore, the total quantity

Table 2. Effects of environmental conditions (EC1 and EC2), nestling sex, and rank (first hatched versus later hatched) on the relationship between body mass index and coloration (top), and on the relationship between response to phytohaemaglutinin (PHA) challenge and coloration (bottom). General linear mixed models included the variable 'age' as a fixed effect and the variable 'nest' as a random effect

Dependent variable	Explanatory	*df*	*F*	*P*	Estimate ± SE
Body mass index					
	Coloration (Col)	1,146	0.04	0.836	–0.001 ± 0.005
	Sex (S)	1,146	109.31	< 0.001	(♀) 0.118 ± 0.011
	Rank (R)	1,146	0.55	0.458	(1st) –0.018 ± 0.025
	EC1	1,146	18.73	< 0.001	–0.011 ± 0.005
	EC2	1,146	3.05	0.083	0.054 ± 0.016
	Col × R	1,146	0.47	0.496	(1st) 0.004 ± 0.007
	Col × EC2	1,146	0.01	0.913	–0.008 ± 0.004
	EC1 × R	1,146	3.74	0.055	(1st) –0.014 ± 0.007
	EC2 × R	1,146	8.22	0.005	(1st) –0.064 ± 0.022
	Col × EC2 × R	1,146	6.83	0.010	(1st) 0.015 ± 0.006
Respnse to PHA*					
	Coloration (Col)	1,80	0.04	0.837	0.856 ± 3.483
	Sex (S)	1,80	0.61	0.437	(♀) 14.458 ± 18.518
	EC1	1,80	7.53	0.007	6.444 ± 9.511
	Col × S	1,80	0.33	0.565	(♀) –2.926 ± 5.068
	Col × EC1	1,80	0.96	0.331	1.707 ± 2.194
	EC1 × S	1,80	3.53	0.064	(♀) 21.956 ± 11.682
	Col × EC1 × S	1,80	4.28	0.041	(♀) –6.825 ± 3.321

*For this analysis, we only considered populations with a minimum sample size of five (see Table S1).

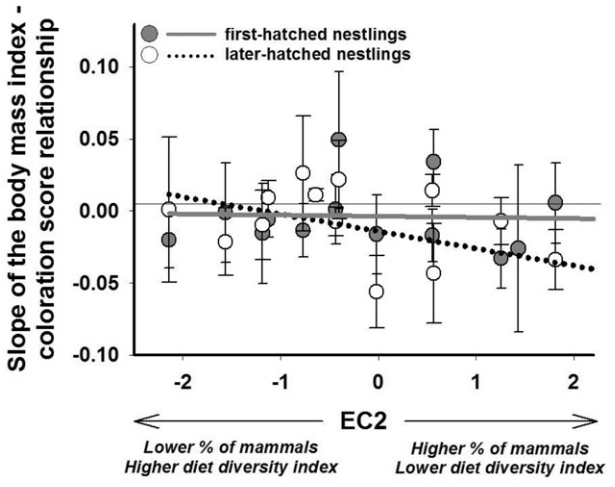

Figure 3. Effect of EC2 (i.e. 'diet conditions') on the body mass index–coloration score relationship (slope ± SE), with nestling rank (first-hatched nestlings, dark-grey symbols; later-hatched nestlings, open symbols). Lines represent fitted linear regressions for first-hatched (bold solid dark-grey line) and later-hatched nestlings (bold dotted line). The fine solid black line represents the null relationship between body mass index and coloration score.

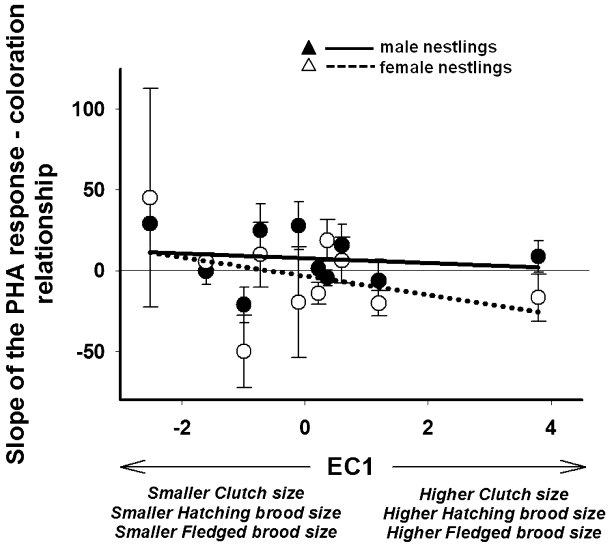

Figure 4. Effect of EC1 (i.e. 'breeding conditions') on the response to phytohaemaglutinin (PHA)–coloration score relationship (slope ± SE), with nestling sex (male nestlings, solid symbols; female nestlings, open symbols). Lines represent fitted linear regressions for male (bold solid line) and female (bold dashed line) nestlings. The fine solid black line represents a null relationship between response to PHA and coloration score.

of carotenoids available probably varied with diet conditions, which may explain the observed variations in nestling coloration. The results are consistent with observations on *C. pygargus* (Sternalski *et al*., 2010) showing that nestling coloration decreased when the percentage of small mammals increased in the diet. More detailed diet analyses are now needed to further investigate a possible trade-off in terms of an energy-rich but carotenoid-poor versus carotenoid-rich but energy-poor diet.

Nestling body mass index also varied with breeding and diet conditions. The relationship between body mass index and diet conditions (EC2) was linear, and independent of sex or nestling rank: in populations or years in which the diet was dominated by mammals and was of low diversity, nestlings were relatively heavier. In raptors, such a pattern is commonly found with the abundance of small mammals strongly and positively impacting upon breeding success and nestling body condition at fledging (Korpimäki, 1984; Wiehn & Korpimäki, 1997; Newton, 1998; Salamolard *et al*., 2000). In contrast, body mass index varied nonlinearly with breeding conditions (EC1) and depended upon nestling rank. Body mass index overall decreased from adverse to good breeding conditions, possibly because larger brood sizes (highest EC1 values) imply more competition. Regardless of the ability of parents to raise more young, sibling competition, which generally results in young engaging in vigorous begging and fights to access the limited food provided by the parents (Mock & Parker, 1997; Wright & Leonard, 2002), is greater in large than in small brood sizes. To acquire and monopolize resources might therefore be more costly for first-hatched nestlings reared within larger rather than smaller broods, which might explain the more pronounced negative relationship between body mass index and high EC1 values in first- rather than in later-hatched nestlings.

In contrast to nestling coloration and body mass index, we did not find that rearing conditions affected circulated carotenoid levels. Geographical or temporal variation in circulated carotenoid levels has been previously reported in different avian species, both in adults and nestlings, and was mostly associated with changes in food availability and type (e.g., Casagrande *et al*., 2006; Arnold *et al*., 2010). Experimental studies involving carotenoid supplementation have shown that circulated carotenoids can increase rapidly after supplementation, whereas integument coloration takes longer to increase (Casagrande *et al*., 2007). Rapid increases of circulated carotenoids after ingestion and rapid mobilization from the bloodstream to alternative functions might thus have precluded us from detecting any variation across population and years.

Immune responsiveness (response to PHA challenge) was influenced by breeding conditions (EC1), depending on nestling rank, but was not affected by diet conditions (EC2). Nestling response to a PHA challenge decreased from adverse breeding conditions and small brood sizes (lowest EC1 values) to intermediate situations. Sibling competition might therefore negatively affect nestling immune responsiveness. Several studies performed on wild (Saino, Calza & Møller, 1997; Hõrak *et al*., 1999) or captive (Naguib *et al*., 2004) birds showed that increasing sibling competition via increased brood size negatively impacted immune function in nestlings, possibly because of a trade-off between growth and immunity (Saino *et al*., 1997; Soler *et al*., 2003). Here, we found a complex (quadratic) relationship between response to PHA and breeding conditions, with the former increasing unexpectedly from intermediate to highest EC1 values. This nonlinear relationship depended mainly on the two populations monitored in 2007 (i.e. IR and MP, see Table S1), but we have no *a priori* reason to remove these data. Indeed, 2007 was a particularly good year, with a peak in vole abundance, resulting in an extremely good breeding performance and large brood sizes. It is therefore possible that despite large brood sizes and high sibling competition, nestlings had so much food that they were able to mount a particularly high response to the PHA challenge. In addition, both under lowest and highest EC1 values, first-hatched nestlings mounted a greater response to PHA than later-hatched nestlings. This suggests that 'extreme' breeding conditions have a stronger influence on the phenotype of the later-hatched nestlings, which are *a priori* more limited and at a competitive disadvantage. Moreover, it emphasizes the value of covering the widest possible range of natural environmental conditions in order to detect such effects, which are nonetheless theoretically predicted (Cotton, Fowler & Pomiankowski, 2004a; Vergara *et al*., 2011).

Rearing conditions and the covariation between body mass index and carotenoid-based coloration

Diet conditions (EC2) modified the relationship between body mass index and coloration, but only in later-hatched nestlings. The slope of the body mass–coloration relationship was close to zero, irrespective of EC2, for first-hatched nestlings, but depended on EC2 for later-hatched nestlings, with the relationship becoming negative with high EC2 values. When diet was dominated by mammals, a negative association between coloration and body mass index became apparent. This observation is consistent with the hypothesis of a trade-off in terms of food energy

versus carotenoid content, particularly when birds feed on mammal prey that are energy rich but carotenoid poor (Goodwin, 1984). In raptors such as harriers, which may specialize on small and large mammals, the condition dependence of carotenoid-based colour traits displayed by nestlings stem, therefore, from the energy/carotenoids ratio occuring in the different types of prey consumed by the nestlings.

Rearing conditions and the covariation between immuneresponsiveness and carotenoid-based coloration

Breeding conditions (EC1) modified the immune responsiveness–coloration relationship, depending on nestling sex. In males, the immune relationship was unrelated to coloration, irrespective of environmental conditions (EC1 or EC2). In females, however, this relationship changed from negative (under good breeding conditions) to null (under adverse breeding conditions). Under good breeding conditions (highest EC1 values), both hatched and fledged brood sizes are larger, indicating that brood competition levels modulate the potential trade-off for the allocation of carotenoid towards immune responsiveness versus coloration in female, but not in male, nestlings. This suggests different carotenoid allocation strategies between nestling sexes, as has previously been proposed (Sternalski *et al*., 2010, 2012), and possibly a sex-specific resolution of the trade-off between growth and immunity in developing nestlings (e.g. Soler *et al*., 2003; Brommer, 2004). Under high brood competition levels and carotenoid limitation, female nestlings may primarily invest carotenoids in signalling (coloration) rather than in immunity, thereby resulting in the negative immune responsiveness–coloration relationship, as we observed. In contrast, irrespective of the environmental conditions, males were more coloured and circulated more carotenoids than females. This suggests, first, that males were less carotenoid-constrained than female nestlings, and second, that males may always prioritize carotenoid allocation towards coloration, resulting in a constant null relationship, irrespective of breeding conditions. Further studies, and in particular experimental studies, would be needed to explore sex-specific carotenoid allocation strategies under contrasting breeding conditions.

Rearing conditions and carotenoid-based signaling in harrier nestlings

Environmental heterogeneity is a reflection of the variation of a wide range of factors, including food supply, adverse weather conditions, parasites, and predators or competitors, and might strongly affect the expression of individual phenotypes (Vergara *et al*., 2011, 2012). In this study we used breeding and diet conditions to characterize environmental heterogeneity and varying rearing conditions experienced by nestlings under contrasting habitats or years. However, breeding conditions (e.g. laying date, clutch size, and fledged brood size) reflect both the environmental conditions and individual parental quality or experience, which may therefore also affect the expression of nestling phenotype. It is somewhat difficult to disentangle environmental effects from those related to individual quality on the expression of nestling phenotype. Our aim here was not to tease apart these interrelated effects, so we must remain cautious about the interpretation of our results because both environmental heterogeneity and parental quality are likely to have contributed to the observed contrasting breeding and diet conditions.

Importantly, we have shown that there is broad variation in signalling, immunity, and carotenoids among populations depending on the context, and that this variation is usually not taken into account. Breeding and diet conditions influenced not only nestling phenotype expression in wild *C. aeruginosus* nestlings, but also the covariation between carotenoid-based trait expression and indicators of individual quality, in terms of body mass index and immune responsiveness. In particular, diet conditions modified the condition-dependence of carotenoid-based traits, possibly because of differences in the energy/carotenoids ratio of prey items consumed by nestlings. In addition, we showed that the influences of breeding or diet conditions were complex, as some relationships were nonlinear or depended on levels of within-brood competition and competitive ability within broods. As in previous studies performed with adults (Martínez-Padilla *et al*., 2010; Vergara *et al*., 2011, 2012), our study highlights that different phenotypes or even associations of traits are found under contrasting environmental conditions. Therefore, whenever possible, studies should be replicated in space and/or time under contrasting natural conditions. Experiments (e.g. brood size manipulations) are now needed to better understand the complex associations between carotenoid-based traits, condition, and immunity, and these should ideally be replicated under contrasting environmental conditions (e.g. Thorogood, Ewen & Kilner, 2011) that capture the broadest possible range of natural variation.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Environmental heterogeneity across space and time.
Table S1. Sample sizes of each nestling phenotypic trait in relation to site and year.
Table S2. Effects of environmental conditions, nestling sex, nestling rank, and their interactions on coloration score, circulated carotenoid levels, body mass index, and response to a PHA challenge.

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