Carotenoid limitation and allocation priorities in asynchronous raptor nestlings

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The evolution and maintenance of conspicuous animal traits and communication signals have long fascinated biologists. Many yellow–red conspicuous traits are coloured by carotenoid pigments, and in some species they are displayed at a very young age. In nestling birds, the functions and proximate mechanisms of carotenoid-pigmented traits are probably different and not as well known as those of adults. Here we investigated how Montagu's harrier (*Circus pygargus*) nestlings within structured families used a limited resource, carotenoid pigments, and whether they used these for increasing coloration (deposition in integuments) or for mounting a response to a phytohaemagglutinin (PHA) challenge, which measures pro-inflammatory potential and aspects of cellular immune responsiveness. We manipulated carotenoid availability, using dietary carotenoid supplementations, and show that when supplemented, nestlings primarily allocated supplemental carotenoids to increase their coloration, irrespective of their sex, but depending of their position within the brood. Responses to PHA challenge were condition-dependent, but depending on carotenoid availability. Moreover, how nestlings allocated carotenoids depended on their rank within the brood, which in turn influenced their level of carotenoid limitation (first-hatched nestlings being less constrained than later-hatched nestlings). We discuss why nestlings would use supplemental carotenoids for increasing bare parts coloration rather than for responding to a PHA challenge, and the potential benefits for doing so in a parent–offspring communication context.   © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, ••, ••–••.

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INTRODUCTION

Evolutionary biologists have long been interested in understanding the functions and proximate mechanisms behind the expression of the conspicuous, exaggerated or brightly coloured traits that many animals display (Darwin, 1871; Andersson, 1994; Espmark, Amundsen & Rosenqvist, 2000; McGraw & Hill, 2006a, b). How these traits evolved and are maintained as reliable quality indicators remains a central issue, which has been extensively studied in adults and in a sexual selection context, but less so in young, despite the fact that conspicuous signals are often displayed at a very young age.

In birds, carotenoid-pigmented traits are amongst the commonest coloured traits involved in social interactions (McGraw & Hill, 2006a) and have received particular attention for two main reasons. First, carotenoids are not synthesizable by vertebrates, but must be ingested and are consequently a diet-limited resource (Goodwin, 1984; Olson & Owens, 1998), particularly in species with food sources that are lacking in carotenoids (e.g. Sternalski *et al.*, 2010). Secondly, it has been proposed that carotenoids have important health-related physiological functions (Chew & Park,

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2004) such as immuno-stimulation (Blount *et al.*, 2003; Faivre *et al.*, 2003), although not all carotenoids may have similar immuno-enhancing properties (e.g. Fitze *et al.*, 2007). Individuals may therefore use available carotenoid pigments for colouring ornamental traits or for self-maintenance needs, the resulting allocation trade-off ensuring honest signalling (McGraw & Ardia, 2003; Mougeot *et al.*, 2007). Indeed, carotenoid-based traits have often been shown to be condition-dependent indicators of 'quality', providing information about the condition and/or immune status and health of their bearers (e.g. Mougeot, 2008; Mougeot *et al.*, 2009; but see also Gonzalez *et al.*, 1999). To date, carotenoid allocation has been mostly studied in a sexual selection context in adult birds (e.g. Andersson, 1994; but see also Metzger & Bairlein, 2011 for alternative functions). In young birds, the functions and proximate mechanisms of carotenoid-based traits are probably different and less well understood (Kilner, 2006). A function in parent–offspring communication is possible, with coloration informing parents about an offspring's need (Kilner, 1997), quality, or value (Saino *et al.*, 2000), allowing parents to adjust their feeding and caring efforts (Lyon, Eadie & Hamilton, 1994). Alternatively, these traits might also already exist in nestlings but acquire their signalling functions only later, at an adult stage and as quality indicators in a sexually selection contex. To assess how and why carotenoid-pigmented traits evolved in nestlings, it is necessary to better understand the proximate mechanisms behind carotenoid allocation to colourful displays. Of particular interest is whether nestlings use supplemental carotenoids for carotenoid-based signalling or for self-maintenance needs and immune responsiveness.

In altricial birds, such as raptors, hatching asynchrony is common, creating marked asymmetries in size and development according to hatching order. This may create different castes of progeny (Magrath, 1990) differing in growth, immunity, mortality, and ultimately fitness (Saino, Calza & Møller, 1997; Saino *et al.*, 2001). The phenotypic handicap of hatching asynchrony therefore exerts a profound influence on the outcome of sibling competition. Older, first-hatched nestlings consistently make better competitors by virtue of their larger size, whereas later-hatched chicks are usually the poorest competitors and typically grow more slowly or die sooner (Magrath, 1990; Arroyo, De Cornulier & Bretagnolle, 2002). Sibling competition for parental attention could thus also drive the evolution of conspicuous visual signals in altricial birds (Soler & Avilés, 2010).

Our aim here is to better understand the mechanisms of carotenoid-pigmented traits in nestlings within structured families (i.e. with marked brood hierarchies). We studied how wild nestlings used carotenoids depending on their availability, which was manipulated using dietary carotenoid supplementations randomly assigned within broods. We studied Montagu's harrier (*Circus pygargus*), a medium-sized, sexually dimorphic raptor species (281 ± 28 and 340 ± 31 g for males and females, respectively; Millon, Arroyo & Bretagnolle, 2008) with hatching asynchrony and facultative siblicide. Brood reduction is mainly due to chick starvation, and, to a lesser extent, to aggression between chicks. Both adults and nestlings of this species display yellow carotenoid-pigmented bare parts (Mougeot & Arroyo, 2006; Sternalski *et al.*, 2010), similar to other raptor species (e.g. Costantini *et al.*, 2007). Montagu's harriers often specialize on mammal prey such as voles, which are energy-rich but carotenoid-poor (Goodwin, 1984; Sternalski *et al.*, 2010), so nestlings are often carotenoid limited. We measured nestlings' responses to a phytohaemagglutinin (PHA) skin test, a test routinely used in bird field studies (Smits, Bortolotti & Tella, 1999; Demas *et al.*, 2011) that measures an individual's pro-inflammatory potential (Vinkler, Bainova & Albrecht, 2010) and some aspects of cellular immune responsiveness (see Martin *et al.*, 2006; Tella *et al.*, 2008). PHA responses are carotenoid-dependent (carotenoid supplementations can enhance responsiveness; Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2004) and indicative of prospective fitness (Møller & Saino, 2004; Cichon & Dubiec, 2005; Moreno *et al.*, 2005). The PHA test is therefore useful to investigate carotenoid use when this resource is limited. We further tested whether hatching order (first-hatched versus later-hatched nestlings within the brood) influenced this carotenoid use, in addition to or in interaction with the carotenoid supplementation treatment. We predicted intra-brood competition to be lower for first-hatched than for later-hatched nestlings within a brood (Arroyo, De Cornulier & Bretagnolle, 2002). In our study species, female nestlings are also heavier than male nestlings, so we predicted greater competitive ability (greater food access, lower intra-brood competition constraints) as well as higher metabolic needs for females, because of their larger body size (Fargallo *et al.*, 2002, 2003; Rowland *et al.*, 2007). We therefore expected hatching order, and also possibly nestling sex, to influence levels of carotenoid limitation and thus carotenoid allocation strategy (with first-hatched nestling being less constrained than other nestlings). By experimentally manipulating carotenoid availability, we tested whether nestlings preferentially used supplemental pigments for colouring bare parts or for responding to a PHA challenge, and whether these carotenoid allocation rules depended on carotenoid supplementation levels, hatching order, and nestling sex.

MATERIAL AND METHODS

STUDY AREA AND FIELD EXPERIMENT

The experiment was conducted in June–July 2006, in an intensive agricultural area of *c*. 450 km^2 located in central-western France (46°15′N, 0°30′W; see Supporting Information S1 for more details). In our study area, Montagu's harriers specialize on a mammal prey, the common vole *Microtus arvalis* (Salamolard *et al*., 2000). This species is a cyclical prey item that exhibits inter-annual variation in abundance (Millon and Bretagnolle 2005; Lambin *et al*., 2006). When voles are abundant, Montagu's harriers feed almost exclusively on this prey, which is energy-rich but carotenoid-poor (Goodwin, 1984; see Casagrande *et al*., 2006 for data on carotenoids content in another vole species, *Microtus savii*). When voles become scarce, as in 2006, they feed more on alternative prey such as passerine birds and insects (Salamolard *et al*., 2000), which are richer in carotenoids but poorer in calories (Goodwin, 1984).

Montagu's harrier nests were searched for with a constant effort during the pre-laying period, and most of them were visited first during egg-laying or incubation. We measured and weighed eggs to estimate hatching date (Millon *et al*., 2008) and revisited nests as soon as possible after hatching to start the experiment. Within a brood, chicks were ranked according to their hatching order and were head-marked using a non-toxic marker pen to allow identification, and later individually ringed. Nests were visited 3–8 times during the nestling period to assess breeding success and collect reproductive data. The experimental birds from this study were a sub-sample from the experiment reported by Sternalski *et al*. (2010), in which changes over time in coloration and plasma carotenoid levels were studied (but not response to PHA and variations according to nestling hatching order). Here, we only considered nestlings for which PHA response was measured (i.e. 74 nestlings from 50 nests), and measurements made upon last visit, closest to fledging.

Carotenoid supplementation effects were tested using a within-brood experimental design (Sternalski *et al*., 2010). Within a given brood, treatment (control or supplemented) was randomly assigned to the (older) first-hatched chick. Hatching order (hereafter referred as to rank) was categorized using two classes (first-hatched chick and later-hatched, younger, chicks) following Arroyo, De Cornulier & Bretagnolle (2002). Other treatments were then sequentially assigned to the remaining nestlings according to their rank within the brood (alternating supplemented and control nestlings with decreasing rank). Within a brood, we thus had control (non-supplemented) and treated (supplemented) nestlings, the treatments being homogenized by hatching order, irrespective of nestling sex.

For supplementations, carotenoids were provided directly in the crop with a flexible syringe as a semi-liquid form of Oro Glo, a commercially available product from Kemin industry (France SRL, Nantes, France). To avoid photo-oxidation of pigments, the solution was kept in a refrigerated bag and in opaque container until given to chicks. The solution contained lutein and zeaxanthin, the two main xanthophyll pigments circulated by the nestlings of this species, in similar proportion to those found in nature (see Sternalski *et al*., 2010; see Supporting Information S1).

Our aim was to supplement nestlings three times, between 10 days of age and fledging (chicks less than 10 days old were considered too young to receive treatment), providing supplementations once every 5 days, with nestlings receiving increasing amounts of carotenoids of 11, 22 and 33 mg, upon first, second, and third visits (when chicks were *c*. 15, 20, and 25 days old), respectively. However, due to fieldwork constraints and natural age differences between nestlings within a given brood, some of the experimental nestlings received two instead of three supplementations, and therefore different carotenoid supplementation doses over a different time course. Within the treated groups, 13 nestlings received two supplementations (total dose of 49 ± 6 mg carotenoids, at 16 ± 3 and 21 ± 3 days of age, corresponding to 0.18 and 0.16 mg g^{-1}, for 21-day-old male and female nestlings, respectively) and 23 nestlings received three supplementations (total dose of 69 ± 10 mg carotenoids, at 13 ± 1, 18 ± 2 and 22 ± 2 days of age, corresponding to 0.25 and 0.23 mg g^{-1} for 21-day-old male and female nestlings, respectively). We thus considered two carotenoid supplementation levels, *a posteriori* (hereafter referred as to 2SUP and 3SUP treatments), according to number of supplementations and doses that nestlings really received (see Supporting Information S1 for detailed sample sizes). Little is known about daily quantities of carotenoids consumed by harriers in their natural diets. We therefore chose supplementation doses following a previous study conducted by Casagrande *et al*. (2007) on common kestrel nestlings (*Falco tinnunculus*), which have a body mass and circulated carotenoids similar to those of Montagu's harriers (Sternalski *et al*., 2010). In addition, as we found no effect of carotenoid treatment on nestling condition index (see below), we are confident that those doses were not detrimental to nestlings.

Upon first visit (before treatment, nestling age: 14 ± 2 days), we took a blood sample and measured (1) body mass (with a Pesola scale, to the nearest 1 g), (2) wing length (with a ruler, to the nearest 1 mm), (3)

tarsus length (with a calliper, to the nearest 0.1 mm) and (4) bare-parts (cere and tarsi) coloration (with a colorimetric chart, see below). When chicks were close to fledging (upon last nest visit, nestling age: 26 ± 2 days), we took another blood sample and measured again body mass, wing length, tarsus length and bare-parts coloration. We then also measured response to a PHA challenge (see below).

Cere and tarsi coloration was measured by direct comparison with a yellow–orange colorimetric chart (Yolk Colour Fan Roche, Neuilly-sur-Seine, France) under shaded light conditions, a method previously used and validated for Montagu's harrier nestlings (see Sternalski *et al*., 2010). The selected colours were characterized by tri-stimulus values of the CIE-1931-standard colorimetric system (Faivre *et al*., 2001). Cere and tarsi colour scores ranged from 1 (very pale yellow) to 6 (bright yellow), were highly repeatable ($R > 0.92$), and were strongly and positively correlated (mixed model with nest as a random effect: $F_{1,46} = 233.59$, $P < 0.001$, $N = 74$; slope $\pm$ SE $= 0.823 \pm 0.054$). As we were interested in the overall carotenoid-based coloration of nestlings (and the overall amount of pigments used), we summed cere and tarsi scores to obtain a total nestling coloration score indicative of overall carotenoid-based coloration.

Blood was taken from the brachial vein using heparinized capillaries, kept refrigerated (0–5 °C) and centrifuged at 10 000$\boldsymbol{g}$ within 4 h of collection. Plasma samples were stored at –20 °C until analysis. Pellets were used to genetically sex nestlings, following Fridolfsson & Ellegren (1999).

CIRCULATING CAROTENOIDS

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$\boldsymbol{g}$ for 5 min to precipitate the flocculent proteins. The optical density of the supernatant was examined at 450 nm using microtitre plates and a Biotek Powerwave XS2 (Winooski, VT, USA) spectrophotometer. Plasma carotenoid concentrations were calculated using a lutein standard curve (Extrasynthese, ref. 0306 S), the main pigment circulated by nestling Montagu's harriers (Sternalski *et al*., 2010). Repeatabilities within and between plates, estimated from a random subset of samples measured twice, were high (intraplate: $F_{14,15} = 26.3$, $P < 0.001$, $r = 0.92$; inter-plate: $F_{54,55} = 10.3$, $P < 0.001$, $r = 0.83$).

ASSESSMENT OF RESPONSES TO A PHA CHALLENGE

We used the PHA skin test to measure some aspects of nestlings' cellular immunity and pro-inflammatory potential. This test 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 (but see Supporting Information). Each nestling was injected with 0.8 mg PHA (SIGMA L-8754) suspended in 0.1 mL phosphate-buffered saline at a marked site on one wing web. We measured web thickness at 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 injection and 24 h after injection. Both initial ($r = 0.90$, $F = 29.09$, d.f. = 73, $P < 0.001$, $N = 222$) and final measurements ($r = 0.92$, $F = 35.33$, d.f. = 73, $P < 0.001$) 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 (see Supporting Information S1).

STATISTICAL ANALYSES

All statistical analyses were performed using SAS 9 (SAS, 2001). We used generalized linear mixed models with normal error distributions and identity link function (Mixed procedure, SAS) to test the effect of nestlings' sex, rank, and treatment on coloration, circulating carotenoids, PHA responses, and condition index (see below). The carotenoid treatment (i.e. control vs. supplemented nestlings) was a three-classes categorical variable (hereafter 'NSUP'), depending on the number of supplementations received: 0SUP (control nestlings), 2SUP (supplemented nestlings with two supplementations), and 3SUP (supplemented nestlings with three supplementations). All models included the variable 'nest' as a random effect to take into account the non-independence of nestlings from the same brood (mean $\pm$ SD: 2.7 ± 1.1 of study nestlings per brood). This random effect was always significant, and therefore was maintained in models. When analyses were conducted on first-hatched nestlings only (one per brood), we used general linear models (GLM procedure, SAS, 2001). The single chick from the three nests with only one chick was classified as first-hatched as brood reduction in raptors generally only affects the youngest chicks (Viñuela, 1999). When condition index was included in models as an explanatory variable, it was calculated using the residuals of a general linear model (GLM procedure, SAS, 2001) of log-transformed body mass on age, age^2 (quadratic relationship with age), log-transformed wing, and tarsus length, as indices of nestling size. Initial models included sex, 'NSUP', rank, and all the

interactions between these explanatory variables, with non-significant ($P = 0.05$ level) terms being removed, starting with interactions, following a backward stepwise procedure, until only the significant explanatory variables or interactions were retained in the models. The Satterthwaite correction was used to approximate the degrees of freedom. 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 also used generalized linear mixed models (as above) to investigate the relationships between responses to PHA and other variables. All tests are two-tailed.

RESULTS

Before the first carotenoid supplementation (nestling age: 14 ± 2 days), coloration (mixed model; treatment: $F_{1,46} = 0.02$, $P = 0.894$), circulating carotenoids (treatment: $F_{1,40} = 0.16$, $P = 0.694$), and condition (body mass corrected for age and size; treatment: $F_{1,45} = 0.95$, $P = 0.335$) did not differ significantly between control and supplemented nestlings (2SUP and 3SUP combined). Prior to treatment, nestling rank had no effect on either coloration ($F_{1,46} = 0.57$, $P = 0.453$) or carotenoid levels ($F_{1,40} = 0.44$, $P = 0.512$). However, condition index marginally varied with nestling rank ($F_{1,45} = 3.58$, $P = 0.065$), with first-hatched nestlings being in relatively better condition [least square means (LSMs) ± SE of 0.03 ± 0.02] than later-hatched nestlings (LSMs of –0.01 ± 0.02), although this difference disappeared when nestlings were close to fledgling (see below).

EFFECT OF SUPPLEMENTATIONS ON COLORATION, CAROTENOID LEVELS, CONDITION INDEX, AND RESPONSES TO PHA CHALLENGE

We first investigated supplementation effects on each study parameter separately, and whether these effects differed according to nestling rank or sex. Prior to fledging, variation in nestling coloration was explained by nestling rank ($F_{1,43} = 5.27$, $P = 0.027$), sex ($F_{1,43} = 5.44$, $P = 0.024$), and 'NSUP' ($F_{2,43} = 17.22$, $P < 0.001$), but was not explained by any interaction between these factors (all $P > 0.28$). Carotenoid supplementation thus increased nestling coloration, but similarly so in nestlings that received two or three supplementations (post-hoc test; see Fig. 1A). Overall, male nestlings were more coloured than female nestlings (LSMs of 8.31 ± 0.35 and 7.72 ± 0.29, respectively), and first-hatched nestlings were more colored than later-hatched nestlings (Fig. 2A).

Variation in circulating carotenoid levels was explained by nestling rank ($F_{1,46} = 7.45$, $P = 0.009$),
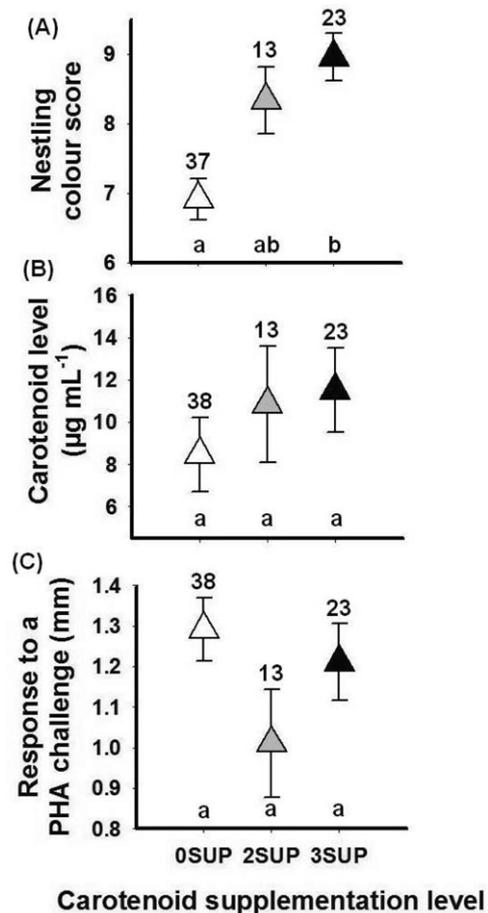

Figure 1. Differences between supplementation groups (non-supplemented = 0SUP: open triangles; 2SUP: grey triangles; 3SUP, filled triangles) in (A) nestling colour score (see Methods), (B) circulating plasma carotenoid levels, and (C) response to a PHA challenge (wing web swelling). Data are expressed as least mean squares ± SE, with sample sizes (number of nestlings) indicated above bars. Pairwise comparisons: two different letters indicate a significant ($P = 0.05$ level) difference between groups.

but not by 'NSUP' ($F_{2,46} = 1.64$, $P = 0.205$), sex ($F_{1,46} = 0.01$, $P = 0.927$), or any interaction between these factors (all $P > 0.47$). First-hatched nestlings had more circulating carotenoids than later-hatched nestlings (Fig. 2B).

Variation in condition index (body mass corrected for age and size) was explained by sex ($F_{1,46} = 6.09$, $P = 0.017$), but not by nestling rank ($F_{1,46} = 0.64$, $P = 0.429$), 'NSUP' ($F_{2,45} = 0.02$, $P = 0.979$), or any interactions between these factors (all $P > 0.23$). Female nestlings were in relatively better condition (LMSs of 0.015 ± 0.016) than male nestlings (LMSs of –0.032 ± 0.018).

Responses to PHA challenge varied according to nestling rank ($F_{1,45} = 5.76$, $P = 0.021$), but not sex
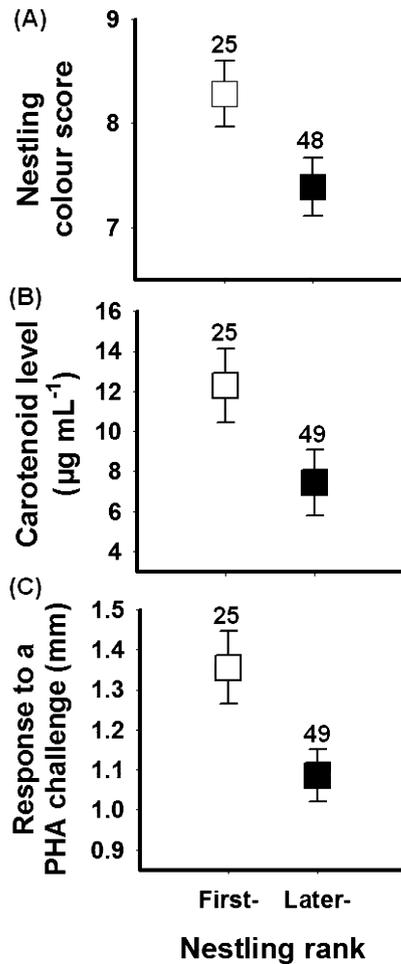
Figure 2. Differences according to nestling rank (first-hatched: open squares and later-hatched nestlings: filled squares) in (A) nestling colour score (see Methods), (B) circulating plasma carotenoid levels, and (C) response to a PHA challenge (wing web swelling). Data are expressed as least mean squares ± SE (and therefore account for treatment and effects other than rank), with sample sizes (number of nestlings) indicated above bars.

Figure 3. Relationship between circulating carotenoid levels and condition index (body mass corrected for age and size) in relation to nestling rank (first-hatched nestlings: filled circles; later-hatched nestlings: open circles). Lines represent fitted linear regressions for first-hatched (thick line) and later-hatched (fine line) nestlings, irrespective of carotenoid treatment.

($F_{1,45} = 0.24$, $P = 0.625$), 'NSUP' ($F_{2,44} = 2.51$, $P = 0.093$), or any interaction between these factors (all $P > 0.23$). First-hatched nestlings mounted greater responses to PHA than later-hatched nestlings (Fig. 2C).

CONDITION INDEX, CIRCULATING CAROTENOID LEVELS AND RESPONSIVENESS TO PHA

As both circulating carotenoids and responses to PHA can be condition-dependent (Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2004), we further tested whether these variables were related to nestlings' condition index, and whether the relationships dif-
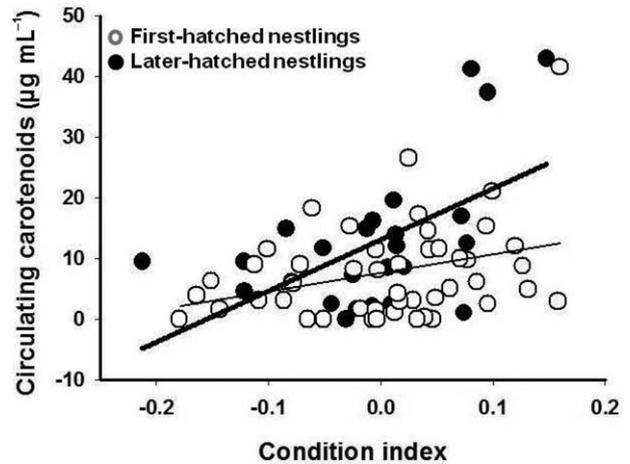
fered between treatment groups or according to nestling sex or rank. For these analyses, we considered only supplemented (2SUP, 3SUP) versus non-supplemented (0SUP) nestlings, because we had no evidence of dose-dependent effects in our sample (non-significant differences between nestlings that received two versus three supplementations; see above).

Variation in circulating carotenoid levels was explained by nestling rank ($F_{1,43} = 12.19$, $P = 0.001$) and by condition index, in interaction with nestling rank (condition: $F_{1,43} = 19.44$, $P < 0.001$; condition × rank interaction: $F_{1,43} = 7.31$, $P = 0.010$), but was not explained by sex ($F_{1,46} = 0.01$, $P = 0.927$), treatment ($F_{1,46} = 2.30$, $P = 0.136$), or any other interactions between these variables (all $P > 0.10$). First-hatched nestlings circulated higher carotenoid levels than later-hatched nestlings (LSMs of $13.43 ± 1.63$ and $7.67 ± 1.39$ µg mL^{-1}, respectively). Circulating carotenoids were overall positively related to condition index (slope ± SE: $22.26 ± 11.44$), this relationship being steeper in first- (GLM: $F_{1,24} = 14.48$, $P < 0.001$; slope ± SE: $91.07 ± 23.93$; Fig. 3) than in later-hatched nestlings (mixed model: $F_{1,25} = 5.49$, $P = 0.027$; slope ± SE: $26.01 ± 11.10$; Fig. 3).

Response to PHA was explained by nestling rank ($F_{1,44} = 6.96$, $P = 0.011$) and marginally by condition index (condition: $F_{1,44} = 3.94$, $P = 0.053$), but it was not explained by sex ($F_{1,46} = 0.24$, $P = 0.623$), treatment ($F_{1,46} = 1.82$, $P = 0.184$), or any interaction between other factors (all $P > 0.12$). Response to PHA increased slightly with condition index (slope ± SE: $0.09 ± 0.65$) and first-hatched nestlings mounted
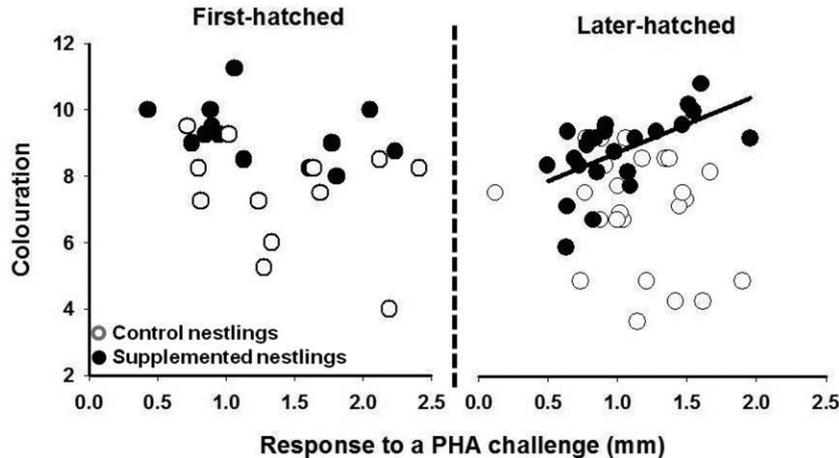
Figure 4. Relationship between coloration and response to PHA (wing web swelling) according to nestling rank (left panel: first-hatched nestlings; right panel: later-hatched nestlings) and carotenoid treatment (non-supplemented: open circles; carotenoid-supplemented: filled circles). Lines represent fitted linear regressions for non-supplemented (dotted lines) and supplemented (solid lines) nestlings.

greater response to PHA than others (LSMs of 1.38 ± 0.09 and 1.09 ± 0.06 mm, respectively).

CAROTENOID ALLOCATION: BARE PARTS COLORATION VERSUS PHA RESPONSE

We further investigated the relationship between nestlings' coloration and response to PHA challenges, and whether it depended on treatment, nestling rank, sex, or their interactions. The relationship between nestling coloration and response to PHA marginally varied according to nestling rank with a two-way interaction (rank × response to PHA; $F_{1,42} = 3.57$, $P = 0.065$), but did not vary with any other interactions (all P values > 0.10).

In first-hatched nestlings, variation in coloration was explained by treatment (GLM; $F_{1,24} = 12.73$, $P = 0.002$; mean ± SE: 7.44 ± 0.47 and 9.29 ± 0.24 for control and supplemented nestlings, respectively; Fig. 4), but not by response to PHA (although there was a negative trend: $F_{1,24} = 2.95$, $P = 0.099$), sex ($F_{1,24} = 1.05$, $P = 0.315$), or any interaction between these variables (all $P > 0.45$).

In later-hatched nestlings, variation in coloration was marginally explained by the treatment × response to PHA interaction (mixed model: treatment: $F_{1,23} = 23.45$, $P < 0.001$; response to PHA: $F_{1,23} = 0.79$, $P = 0.383$; treatment × response to PHA interaction: $F_{1,23} = 3.92$, $P = 0.059$), but not by nestling sex ($F_{1,25} = 2.39$, $P = 0.135$) or any interactions between these factors (all $P > 0.10$). In control nestlings, coloration was not related to response to PHA ($F_{1,8} = 0.11$, $P = 0.747$; slope ± SE: 0.002 ± 0.007; Fig. 4), whereas in supplemented nestlings, this relationship was positive and significant ($F_{1,6} = 10.52$, $P = 0.018$; slope ± SE: 0.021 ± 0.006; Fig. 4).

DISCUSSION

EFFECTS OF CAROTENOID TREATMENT

Carotenoid treatment increased the yellow coloration of nestlings' cere and tarsi, consistent with previous studies on this species (Sternalski *et al.*, 2010) and other raptors (e.g. common kestrels; Casagrande *et al.*, 2007). Carotenoid-based coloration expression of wild Montagu's harrier nestlings was therefore limited, consistent with the carotenoid-limitation hypothesis (Blount *et al.*, 2004). Carotenoid use might be limited by physiological thresholds (Alonso-Alvarez *et al.*, 2004), such as the ability to absorb and/or transport carotenoids (Surai, 2002) or the ability to accumulate pigments in skin. The dose and timing of the supplementation might also affect pigment use (Fitze, Tschirren & Richner, 2003), but we did not find differences in coloration between nestlings that were supplemented two or three times, which received 49 vs. 69 mg of carotenoids when 13 or 16 days old, respectively. We therefore had no evidence that supplementation effects were dose- or timing-dependent in these nestlings (but see Casagrande *et al.*, 2007).

Regardless of treatment, male nestlings were more colored than female nestlings, as shown previously in this species (Sternalski *et al.*, 2010) and in adult raptors (e.g. American kestrels *Falco sparverius*: Bortolotti *et al.*, 1996), in which carotenoid-based coloration most likely has a function in sexual display (Mougeot & Arroyo, 2006). Males might therefore use

carotenoids for coloration more than females, which may store these pigments in organs for using later on (e.g. Surai, 2002). Sexually selected traits might already exist in nestlings but acquire their signalling functions only later in life, and bare parts coloration could have a sexual signalling function in adult Montagu's harrier (Mougeot & Arroyo, 2006). These different carotenoid allocation strategies could also involve sex hormones, and their modulatory effect on circulating carotenoids (Laaksonen *et al*., 2008) or carotenoid deposition in tissues (Bjerkeng *et al*., 1999). Specifically, nestling males could circulate more testosterone than females, a hormone that influences carotenoid availability and allocation (Blas *et al*., 2006; Laaksonen *et al*., 2008; Alonso-Alvarez *et al*., 2009). In addition, as females are heavier and grow faster than males, they might have to cope with greater free radical production associated with accelerated growth (Surai & Speake, 1998) and thus may need more carotenoids for combating oxidative stress or for physiological detoxification than males.

In contrast to coloration, carotenoid treatment did not increase circulating carotenoid levels to any great extent, as previously found in other nestlings (e.g. great tit *Parus major* and blue tit *Cyanistes caeruleus*, Biard, Surai & Møller, 2006). Casagrande *et al*. (2007) showed that circulating carotenoids increase rapidly after supplementation, while integument coloration takes longer to increase. We may not have found an effect on circulating carotenoid levels because blood was taken too long after the last supplementation (mean ± SE: 4 ± 1 days), with carotenoids having already been used for coloration and/or alternative functions, or stored in organs such as liver and fat (Surai, 2002).

Carotenoid supplementations did not affect responses to the PHA skin test. Previous studies also found a lack of effect (e.g. Biard, Surai & Møller, 2006; Fitze *et al*., 2007), but others did find that carotenoid supplementation enhanced PHA responses (e.g. McGraw & Ardia, 2003). Therefore, Montagu's harrier nestlings used supplemental carotenoids for colouring bare parts but not for responding more to a PHA challenge. Our result may also be explained by the type of carotenoids used in dietary supplementation. For instance, Fitze *et al*. (2007) demonstrated that nestling great tits supplemented with lutein, zeaxanthin, and β-carotene developed greater response to PHA, in contrast to those supplemented with only the first two xanthophylls, suggesting that only β-carotene had immuno-enhancing properties (Bendich, 1991; see also Navara & Hill, 2003; Cucco *et al*., 2006). It is therefore possible that our supplemented nestlings did not mount greater response to PHA challenge because the xanthophylls they were supplemented with did not have immuno-enhancing

properties, at least considering our specific PHA-test. Despite the lack of a carotenoid supplementation effect on PHA response in this study, further studies should examine simultaneously various aspects of the immune system, in order to better assess an individual's immunocompetence (see Demas *et al*., 2011).

CAROTENOID SUPPLEMENTATION EFFECTS AND HATCHING ORDER

In a dominance hierarchy within broods, as in Montagu's harrier, first-hatched nestlings can control, to some extent, food distribution among nestlings: the amount of food later-hatched nestlings consume is the amount first-hatched nestlings are willing to concede and, eventually, last-hatched nestlings simply take whatever is left by others. Older chicks are therefore largely buffered from environmental and developmental uncertainty by younger chicks, such that the presence of youngest sibs has virtually no effect on the development or fitness of first-hatched chicks (Forbes & Glassey, 2000). Competition between sibs appears harsher for later-hatched nestlings in contrast to first-hatched nestlings. Within structured families with marked hatching asynchrony, carotenoid-based trait expression, as a signal of nutritional status or need, may therefore be modulated by the level of competition between nestlings and thus vary with nestling rank. Accordingly, nestling rank influenced coloration, circulated carotenoid level, and also responses to PHA. First-hatched nestlings were more coloured, had more carotenoids, and mounted greater responses to PHA, irrespective of the carotenoid supplementation level. This indicates that within-brood competition influenced chicks' stress levels and/or resource distribution between nestlings, first-hatched chicks suffering less competitive stress and being less carotenoid limited than others. Later-hatched nestlings might indeed suffer higher level of competitive stress, which in turn might affect level of stress hormones in those nestlings. High levels of stress hormones have been showed to increase oxidative damage (as well as starvation), which could explain higher carotenoid depletion in those nestlings and their paler coloration. Alternatively, Müller, Dijkstra & Groothuis (2003) found a decrease in responses to PHA in relation to hatching order in black-headed gull (*Larus ridibundus*), last-hatched nestlings raising lower responses than first-hatched nestlings; they proposed that lower carotenoid levels in last-laid eggs could explain these differences. Differential micronutrients (such as carotenoids), but also hormone (such as corticosteroids or testosterone) provisioning in relation to laying order have been found in several bird species (e.g. Saino *et al*., 2001; Blount *et al*., 2004) and even raptors (e.g. American

kestrel; Sockman & Schwabl, 2000). Such mechanisms may also exist in the Montagu's harrier, but further research would be needed to confirm this.

CAROTENOID ALLOCATION: COLORATION VERSUS RESPONSIVENESS TO A PHA CHALLENGE

The relationship between coloration and response to PHA was complex, as it depended both on carotenoid treatment and on nestling rank. In later-hatched nestlings, no relationship between coloration and response to PHA was found in control nestlings, but a positive relationship was found in supplemented nestlings (i.e. greater coloration was associated with greater responsiveness). Later-hatched nestlings, suffering more from intra-brood competition for resource distribution (with, to some extent, control of food distribution by first-hatched nestlings), were probably highly constrained in terms of both energy and carotenoid acquisition. In control nestlings, carotenoids appeared therefore to be limited through diet acquisition and the few available carotenoids were allocated to bare parts coloration rather than responsiveness to the PHA challenge. When supplemented, nestlings still increased coloration but were also able to reallocate the supplemental carotenoids for greater responses to the PHA challenge. In contrast, first-hatched nestlings, which control, to some extent, food distribution, were less likely to be carotenoid limited through diet. Accordingly, first-hatched nestlings allocated supplemental dietary carotenoids to coloration rather than to respond to a PHA challenge (coloration but not response to PHA increased with supplementation) independently of carotenoid treatment, suggesting that carotenoids were less limiting for those nestlings (or that they suffer less competitive stress), in contrast to later-hatched nestlings.

Carotenoid limitation might be the result of availability through diet, as was probably the case for later-hatched nestlings, but might also result from a limited physiological capacity to absorb and/or transport carotenoids (Surai, 2002; Tschirren, Fitze & Richner, 2003). Indeed, carotenoids are absorbed and transported by lipoprotein complexes consisting of proteins and lipids (e.g. triglycerides; Stevens, 1996). As lipids are the main energy reserves, a trade-off may occur between lipids being used for energy generation and for absorption and/or transportation of carotenoids (Surai, 2002). Activation of immune function, such as a PHA challenge, increases energy demand and therefore consumption of lipids (see Demas, 2004 for a review). As a consequence, fewer lipids are available for absorption and/or transportation of the ingested carotenoids, which in turn may cause a negative relationship between colour expression and responsiveness to PHA, as the tendency found in first-hatched nestlings (see Fig. 4), which circulated more carotenoids than non-supplemented nestlings. We thus suggest that in Montagu's harrier nestlings, as proposed by Fitze *et al*. (2007), carotenoid allocation towards coloration and responsiveness to PHA depends on nestling rank within a brood and their associated carotenoid limitation levels. Carotenoid-based coloration would therefore reflect an individual's nutritional condition and therefore competitive ability, rather than its response to a PHA challenge.

WHY SHOULD NESTLINGS INVEST MORE IN CAROTENOID-BASED COLORATION?

It is somehow surprising that nestlings used the supplemental carotenoids primarily in favour of coloration. Such a strategy may be 'risky' (i.e. to the detriment of immune responsiveness) so there should be some benefits for nestlings to increase investment in bare parts coloration.

In the Montagu's harrier, parents individually feed their young until aged 10–15 days. Afterwards, they drop the food into the nest, with food access being then controlled by nestling competition. However, for another period of 25 days after fledging (i.e. a period as long as the time spent by nestlings at the nest), parents again feed their young individually (during the post-fledging period, 62% of all deliveries in families with broods were attributed to an individual chick: Arroyo, De Cornulier & Bretagnolle 2002). This period is critical in migratory raptors, such as harriers (Bustamante, 1993, 1995; Bustamante & Negro, 1994; Arroyo, De Cornulier & Bretagnolle, 2002), and carotenoid signals could then play an important role as a signal towards parents. If carotenoid-based coloration has such a function, nestlings might allocate available carotenoid pigments in coloration primarily to gain more parental food provisioning, as supported in other species (Lyon *et al*., 1994; Kilner, 1997). In this context, carotenoid-based traits could indicate the quality and/or food needs of nestlings (Saino *et al*., 2003). If parents respond to nestling coloration by increasing their feeding rate, benefits for nestlings could be two-fold: first, an improved growth and condition index, through direct food acquisition, and secondly, more micronutrients, such as carotenoids, available for responding to immune challenges and for resisting adverse conditions (a nestling's immune response being a trait that depends on nutritional condition; e.g. Saino *et al*., 1997; Alonzo-Alvarez & Tella, 2001). Allocation of supplemental carotenoids in coloured traits might thus allow nestlings to signal to parents their nutritional condition and their competition ability rather than specifically their immune potential.

In conclusion, we have shown here that within a structured family, nestlings of a raptor species use carotenoid pigments, a limited resource, to increase bare parts coloration rather than for responding to a PHA challenge. However, how nestlings invest limited carotenoids to coloration or responses to PHA depended on their rank within the brood (with first-hatched nestlings being less limited than others) which, in turn, influenced their level of carotenoid limitation. Further investigations are needed to assess the benefits of increased carotenoid-based coloration for nestlings in Montagu's harrier, depending on their rank, and more importantly, their possible function as signal toward parents or sibs.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supporting Information S1. Additional information on study area; carotenoid supplementations; sample sizes; assessment of responses to phytohaemagglutinin challenge.

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