

Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment

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Received: 26 September 2005 / Accepted: 2 May 2006 / Published online: 4 July 2006
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Abstract In monogamous species, the value of present reproduction is affected by the current condition of the mate, and females may use male ornaments to evaluate his condition and adjust their level of investment according. Many animals display colour in fleshy structures which may be accurate indicators of quality due to their potentially rapid response to changes in condition. Here we show that in the blue-footed booby, *Sula nebouxii*, male foot colour is structurally (collagen arrays) and pigment based. In 48 h foot colour became duller when males were food deprived and brighter when they were re-fed with fresh fish. Variation of dietary carotenoids induced comparable changes in cell-mediated immune function and foot colour, suggesting that carotenoid-pigmentation reveals the immunological state of individuals. These results suggest that pigment-based foot colour is a rapid honest signal of current condition. In a second experiment, we found that rapid variation in male foot colour caused parallel variation in female reproductive investment. One day after the first egg was laid we captured the males and modified the foot colour of experimental males with a non-toxic and water resistant duller blue

intensive make-up, mimicking males in low condition. Females decreased the size of their second eggs, relative to the second egg of control females, when the feet of their mates were experimentally duller. Since brood reduction in this species is related to size differences between brood mates at hatching, by laying lighter second eggs females are facilitating brood reduction. Our data indicate that blue-footed booby females are continuously evaluating their mates and can perform rapid adjustments of reproductive investment by using dynamic sexual traits. We suggest that this fine-tuned adjustment may be widespread in socially monogamous animals.

Keywords Dynamic trait · Differential allocation · Sexual signal · Carotenoids · Brood reduction

Introduction

In iteroparous organisms, life history predicts that investment in offspring is balanced against the negative effects on future reproduction (Stearns 1992). If increased parental effort in current reproduction negatively affects future reproduction, animals should continuously evaluate the value of their current reproduction and adjust investment accordingly. In monogamous species, the value of present reproduction may be affected by the current condition of the mate (Cunningham and Russell 2000) and, particularly in long-lived animals, females should optimise their reproductive decisions in relation to partner quality and his level of investment (Winkler 1987). Models of sexual selection predict that females should use ornament expression to evaluate male condition

Communicated by Carol Vleck

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(Andersson 1994). Attractive males might provide genes for attractiveness or viability, and/or high parental investment (Andersson 1994). Therefore, females may use male ornaments as indicators of the expected value of current reproduction, and adjust their current investment accordingly (“differential allocation hypothesis”; Burley 1986; Gil et al. 1999; Cunningham and Russell 2000; Sheldon 2000 for a review).

In iteroparous bird species with prolonged biparental care, mate quality probably varies from pair formation to chick feeding, consequently varying the value of current reproduction; in this context, females should continuously evaluate the quality of their partner (de Lope and Møller 1993; Torres and Velando 2003; Saffran et al. 2005). Previous experiments on differential allocation have shown that females modify their breeding decisions after pairing in relation to male attractiveness, adjusting their investment according to static male sexual traits (de Lope and Møller 1993; Limbourg et al. 2004) or artificial ornaments (Burley 1986; Gil et al. 1999). Nevertheless, as the studied traits did not vary during the breeding season, these results may indicate that some decisions are taken after pairing independently of whether females are continuously evaluating male attractiveness. Hence, fine-tuned adjustment of reproductive investment according to changes in mate condition, using ornament expression as cue, has not previously been investigated.

Many animals display colour in fleshy structures which may be accurate indicators of quality due to their potentially rapid response to changes in condition (Lozano 1994; Faivre et al. 2003). The expression of many colours in fleshy tissues results from carotenoid pigments that are deposited in the integuments. Carotenoids cannot be synthesized *de novo* by animals, and instead must be obtained through the diet (Olson and Owens 1998). Since these pigments are antioxidants and immunostimulants, it has been suggested that individuals that invest carotenoids in sexual signalling could not do so without diverting them from the immune and the detoxification systems (Lozano 1994). In recent studies on birds, males exposed to an antigen exhibited diminished beak colour, a carotenoid-dependent sexual signal, suggesting a trade-off in carotenoid allocation between the signal and the immune system (Faivre et al. 2003; Peters et al. 2004; Alonso-Alvarez et al. 2004). Additionally, studies on birds and fish have shown that supplementing carotenoids results in enhanced expression of both the sexual signal and the immune response (Blount et al. 2003; McGraw and Ardia 2003; Grether et al. 2004). Thus, the information expressed by

colourful skin displayed by many animals probably differs from plumage colours, since pigments present in fleshy structures can be mobilized facultatively (Lozano 1994), may reflect more recent physiological events, and have the potential to indicate current physical condition, allowing females to continuously evaluate mate condition.

The blue-footed booby, *Sula nebouxi*, is a socially monogamous seabird with an extended period of pair courtship (Nelson 1978; Osorio-Beristain and Drummond 1998). Males assist females with caring for nestlings and male condition is an important factor for female breeding success (Velando and Alonso-Alvarez 2003). Experimental reduction of paternal effort has a strong negative effect on the condition, and hence, probable future reproduction of females (Velando and Alonso-Alvarez 2003). Therefore, females should adjust investment in relation to current mate condition. During courtship, blue-footed booby males exhibit a bluish foot-web to their mates in a foot-raising dance. Male foot colour varies from bright green to dull blue. Females prefer bright green feet and decrease courtship and pair copulations when mates display dull blue feet (Torres and Velando 2003). Although blue–green integuments in animals have been thought to be structurally based (Auber 1957; Prum and Torres 2003), it has been suggested that foot colour on boobies may be carotenoid dependent (Nelson 1978).

In this study, we first investigated foot colour variation in the blue-footed booby and examined a skin sample to determine if foot colour results from structural and pigmentary mechanisms. We also investigated whether foot colour changes rapidly with male condition by manipulating the amount of food and dietary carotenoids available to courting males kept in captivity. In a second experiment, we tested whether blue-footed booby females adjust their investment according to mate foot colour. One day after the first egg was laid we captured the males and modified the foot colour of experimental males to duller blue, mimicking males in low condition, and measured female investment in the second egg. Blue-footed boobies normally lay two eggs with a mean laying interval of 5 days (Nelson 1978; Drummond et al. 1986). In this species, siblicidal brood reduction is related to size differences between brood mates at hatching (Drummond et al. 1986). Females lay heavier second eggs compared to first eggs when ecological conditions are good, possibly as a mechanism to compensate for the asymmetries among brood siblings, but lay lighter second eggs when the food is limited, facilitating the brood reduction (D’Alba 2000).

Materials and methods

The studies were carried out in the blue-footed booby colony at Isla Isabel, Nayarit, Mexico, from January to March 2003 and 2004.

Skin colour

In 2004, we captured 70 courting males and measured the colour of the left foot in three different web areas using a spectrophotometer (MINOLTA CM-2600d). Reflectance curves were compared at the maximum visual sensitivity area (460–620 nm) of this species (Reed 1987). A fresh sample of foot skin tissue was obtained from a dead courting male that was found in the colony, was washed to remove any spilled blood, and was immediately preserved in liquid nitrogen until analyses in the laboratory. A small piece of this skin was embedded in paraffin, cut, stained with methylene blue and examined with a light microscope. To determine the presence of carotenoids, lipid-soluble pigments were extracted from the coloured skin with heated acidified pyridine and transferred to a solvent solution (hexane/TBME; details in McGraw et al. 2005a). The spectral-absorbance profile of extracted pigments in the region where carotenoids exhibit absorption maxima (400–500 nm; McGraw et al. 2005a) was determined by spectrophotometry.

Effects of food and carotenoids on foot colour and immune response

In 2003, 20 courting males were kept individually in cages placed in the forest, away from heat and noise stress. This method has been used before without any adverse effects on the birds (Osorio-Beristain and Drummond 1988). Males were randomly assigned into four groups (five per group) to perform a two-factorial experiment manipulating the food (no food or 200 g per day of fresh fish, mainly *Sarda sarda*) and dietary carotenoids (no carotenoids or a daily diet of cantaxanthin 0.2 mg, betacarotene 0.2 mg, lutein 0.2 mg, and zeaxanthin 0.05 mg). The duration of the food deprivation treatment (48 h) was within the natural range that blue-footed boobies can be without any food or water (75 h) (Nelson 1978). The amount of fish in the food-supplied (hand-fed) treatments was within the natural range of intake and probably represents twice the amount needed to maintain the basal metabolic rate of male boobies (estimation based on food consumption of cape gannets, *Morus capensis*) (Adams et al. 1991). There are no data on the specific composition of carotenoids in the diet of blue-footed boobies,

yet it is unlikely that the 1.3 mg of carotenoids used here (a mixture of carotenoids regularly added to the diet of domestic birds) was harmful to our experimental birds (Klasing 1988). Food-deprived males lost on average $11 \pm 0.47\%$ (range 10–11%) of their original mass. After 48 h of food deprivation the birds were fed with 120 g of fresh fish every 12 h for 36 h to allow them to recover part of the lost mass.

To estimate foot colour, two measures on the web from the left foot were taken every 12 h using a colorimeter (MINOLTA CR-200) with CIELAB parameters ($L^*a^*b^*$ colour space), a standardized method based on tristimulus values to express colour numerically. In the $L^*a^*b^*$ colour space, L^* indicates brightness, and a^* and b^* indicate the chromaticity coordinates. The saturation of the colour, given by the coordinates a^* and b^* , increases as a^* and b^* absolute values increase. In the experiment, foot colour chromaticity varied from dark blue (negative values of b^* and low absolute values of a^*) to a bright green (positive values of b^* and negative values of a^* ; Fig. 1). CIELAB measures were significantly repeatable (intra-class correlation coefficient, L^* , $r = 0.93$; a^* , $r = 0.91$; b^* , $r = 0.91$; $P < 0.001$ in all cases) and the mean of the two measures was used in the analyses. We measured in vivo cell-mediated immune-response by intradermally injecting phytohemagglutinin (PHA) (Smits et al. 1999). At 36 h after the start of the experiment, we injected 0.2 mg of PHA in 0.1 ml of phosphate buffered saline in the left wing-web of males in captivity. The cell mediated immunity (response of T lymphocytes) was measured as the change in thickness (mm) of the wing web 24 h post-injection (12 h after re-feeding started). Wing-web thickness measures were significantly repeatable, both for initial measures (intra-class correlation coefficient, $r = 0.92$, $P < 0.001$) and for final measures ($r = 0.94$, $P < 0.0001$).

Effects of foot colour modification on egg investment

In 2004, we monitored nest sites daily until the first eggs were laid. Experimental manipulations were carried out one day after the first egg was laid; at this time, males were captured and randomly assigned to the experimental ($n = 26$) or control group ($n = 27$). Prior to the experimental manipulation, foot colour of all captured males was measured using a spectrophotometer (MINOLTA CM-2600d). The foot colour of experimental males was modified with a non-toxic and water-resistant duller blue intensive make-up (Laukrom), mimicking the foot colour of poor condition males (foot colour modification; L^* , 42.9 ± 0.3 ; a^* , -0.9 ± 0.7 ; b^* , -34.5 ± 0.6 ; see Torres and Velando

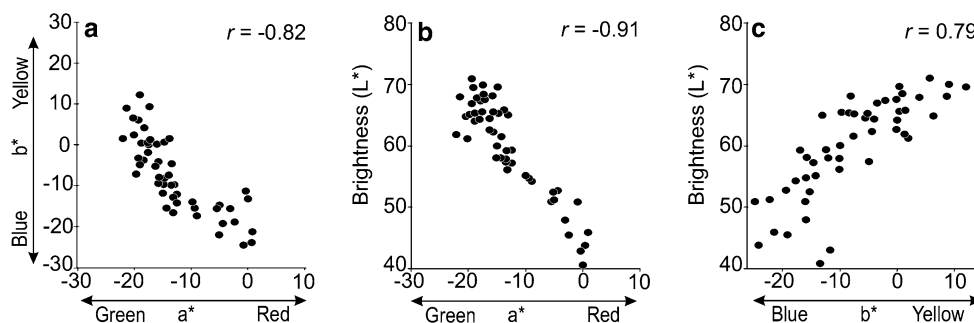


Fig. 1 Relationships between measurements of blue-footed booby (*Sula nebouxii*) foot colour during the food controlled experiment. Foot colour was measured using a standardized method to express colour numerically based on tristimulus values

($L^*a^*b^*$ colour space). **a** Chromaticity coordinates a^* and b^* , **b** Chromaticity coordinate a^* and Brightness (L^*), and **c** Chromaticity coordinate b^* and brightness (L^*)

2003, 2005). This method of colour modification has been used before with no effects on bird behaviour and the artificial colour on experimental males lasts for 5–6 days. The feet of control males was sham coloured (using a crayon in a plastic bag) to imitate the manipulation and handling time of experimental males (details in Torres and Velando 2003, 2005). The nest and the first-laid egg were marked and from day 2 after the manipulation nests were monitored to determine egg volumes and laying intervals. Before the manipulation, foot colour of experimental and control males did not differ (PC1 score, see below; $t_{1, 50} = 1.56$, $P = 0.12$). Laying date and first egg volume did not differ between control and experimental groups (laying date, $t_{1, 52} = 0.96$, $P = 0.33$; egg volume, $t_{1, 52} = 0.12$, $P = 0.89$).

Statistical analysis

Since CIELAB parameters were highly correlated (Fig. 1), a principal component (PC1) was calculated from a factorial analysis with the correlation matrix of these parameters, including all the data measured for each individual studied. The first PC1 accounted for 89.5% of variance with a correlation of 0.95 with L^* , -0.96 with a^* and 0.95 with b^* . PC1-negative values indicate dark blue and PC1-positive values bright green. Thus, the first PC1 was a synoptic descriptor of foot colour and its variation during the experiment. In the food-controlled experiment, colour changes (PC1 changes) were compared by repeated-measures ANOVA, with food and carotenoid treatments as between-subject variables and the repeated measures of foot colour at different times (initial and final) as within-subject factors. The effect of the treatments on colour changes was tested by the significant interaction between treatments and time. The cell mediated immunity, measured as the change in wing-web thick-

ness was transformed to its natural logarithm to meet the requirements of parametric tests and analyzed by ANOVA. The effect of experimental foot colour manipulation on second egg volume was analyzed by ANCOVA, using first egg volume as a covariate. Data are expressed as mean \pm SE.

Results

Skin colour

Male foot colour during courtship varied from dull blue (reflectance peak at 440 nm) to bright green (reflectance peak at 540 nm), with the highest variation in the wavelength area where blue-footed boobies have maximum visual sensitivity (Fig. 2a). Histological analysis of foot skin revealed a thick dermal collagen layer below the epidermis (Fig. 2b). Using a thermochemical extraction with organic solvent transfer, we determined the presence of lipid-soluble pigments in the foot integument of male boobies. The analysis of the spectral-absorbance profile of extracted pigments revealed the presence of four peaks of maximum absorption in the range of carotenoid absorbance (410, 420, 440 and 460 nm, Fig. 2c).

Effects of food and carotenoids on foot colour and immune response

Foot colour of courting males varied with male nutritional condition and dietary carotenoids. After 48 h, foot colour was significantly affected by food supply ($F_{1,16} = 5.88$, $P = 0.028$; Fig. 3a) and dietary carotenoids ($F_{1,16} = 5.02$, $P = 0.037$; Fig. 3a). The interaction between carotenoids and food supply was not significant ($F_{1,16} = 0.001$, $P = 0.98$). Males deprived of food and carotenoids showed duller blue feet (Fig. 3b), in

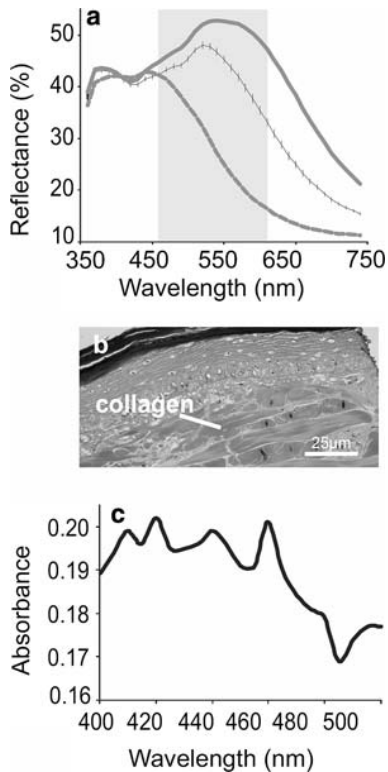


Fig. 2 Foot colour displayed during courtship by blue-footed booby males. **a** Reflectance curves of foot colour of courting males [the brightest (grey line), the duller (dashed line) and the mean \pm SE (black line)]. Shaded area indicates the range of maximum visual sensitivity (460–620 nm) of blue-footed boobies (Reed 1987). **b** Light micrograph of foot skin showing a collagen layer. **c** Spectral-absorbance profile of yellow pigments (probably carotenoids) extracted from foot skin

contrast, males fed with fresh fish and a supplement of carotenoids displayed pale green feet (Fig. 3c). Additional evidence that web colour is a dynamic honest signal of current condition was provided by the fact that the feet colour of food-deprived males reverted to nearly their original colour when the birds were fed during the next 24 h (PC1 change, $F_{1,8} = 33.25$, $P < 0.0001$; carotenoids \times change $F_{1,8} = 0.77$, $P = 0.41$; Fig. 3d).

The manipulation of dietary carotenoids in the blue-footed booby altered the cell mediated immune response (carotenoids treatment, $F_{1,16} = 4.97$, $P = 0.040$, Fig. 4a; food treatment, $F_{1,16} = 0.07$, $P = 0.80$; carotenoids \times food, $F_{1,16} = 0.95$, $P = 0.34$). Furthermore, foot colour was correlated with the cell mediated immune response ($r = 0.44$, $n = 20$, $P = 0.050$; Fig 4b).

Effects of foot colour modification on egg investment

Laying interval between the first and the second eggs did not differ between experimental group

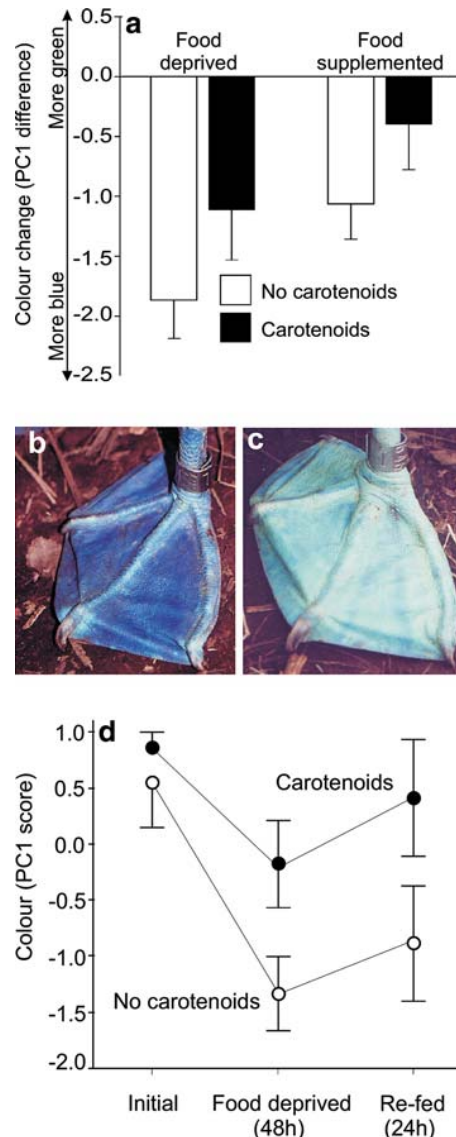


Fig. 3 Foot colour after manipulation of nutritional condition and dietary carotenoids. **a** Difference between initial and final foot colour (mean \pm SE) for the four experimental groups. Examples of foot colour changes after 48 h in the food and carotenoids **b** deprived and **c** supplemented treatments. **d** Foot colour (mean \pm SE PC1 score) at initial, 48 h after food deprivation and 24 h after re-feeding birds without (open circles) and with (filled circles) carotenoids

(4.56 ± 0.29) and control groups (4.64 ± 0.29 days; $F_{1,51} = 0.42$, $P = 0.83$). The volume of second eggs was positively related to the volume of the first egg in the clutch ($F_{1,51} = 27.35$, $P < 0.0001$). After controlling for the effect of the volume of the first egg, females laid significantly smaller second eggs when mated to males with experimentally duller feet compared with second eggs laid by females in the control group ($F_{1,51} = 10.16$, $P = 0.003$; Fig. 5).

Fig. 4 T-cell mediated immunoresponse (change in wing-web thickness, mm) and **a** carotenoid supplementation (mean \pm SE, $n = 20$), and **b** foot colour at the end of the experiment. Data from food-deprived and food supplemented birds without (*open circles*) and with (*filled circles*) carotenoids are shown

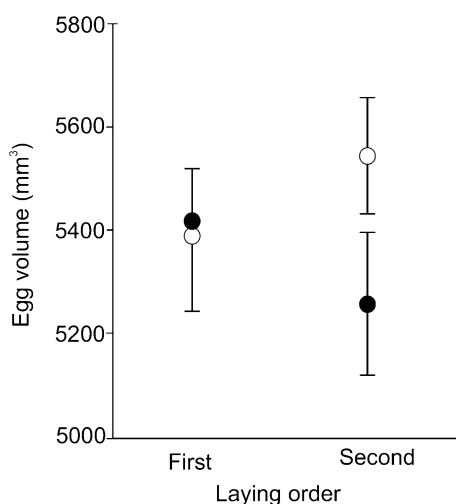
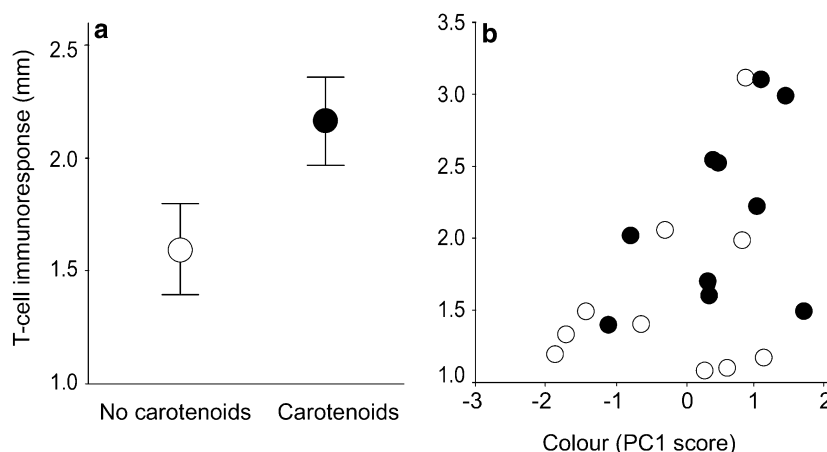


Fig. 5 Egg volume (mean \pm SE) of first and second-laid eggs for experimental [(*filled circles*), $n = 26$] and control [(*open circles*), $n = 27$] clutches. The foot web of males in experimental clutches was modified to a duller blue colour

Discussion

Blue skin colours are one of the most strikingly colourful traits in nature and are considered to be structurally based (Auber 1957; Prumm and Torre 2003). Nevertheless, in the blue-footed booby, we found that integumentary foot colour may result from the combination of structural mechanisms (collagen arrays) and pigments. In this species, previous examination of skin tissue, probably from non-courting birds (the preserved tissue examined was dull grey; Prum and Torres 2003, page 2425), showed that collagen arrays predicted dark blue reflectance spectra (reflectance peak at 410 nm; Prum and Torres 2003), close to the colour of the duller foot in our sample (Fig. 2). In our study, courting males showed a reflectance peak at 540 nm, suggesting that additional factors are involved

in the production of foot colour. We analysed a skin sample from a courting male (reflectance peak at 540 nm), whose preserved tissue still displayed vivid colour. In this sample, the presence of lipid-soluble yellow pigments, probably carotenoids, was determined by acidified-pyridine treatment (MacGraw et al. 2005a). The isolated pigments from our sample showed four peaks of absorbance at the maximum absorbance picks of yellow carotenoids, although HPLC analysis should be used to confidently identify these yellow pigments (K. McGraw, personal communication). These results indicate that in the blue-footed booby, green skin during courtship is probably produced by the combined effects of structural blue and yellow (probably carotenoids) pigments.

In addition, we found that dietary carotenoids influence foot colour. The effect of dietary carotenoids on skin colouration can be due to their direct effect as pigments or alternatively due to their beneficial effects on the individual's health (Blount et al. 2003; McGraw and Ardia 2003). The influence of dietary carotenoids on carotenoid pigmentation has been well established (Hill 2002). Nevertheless, we also found that foot colour in the blue-footed booby is a rapid honest signal of current nutritional condition. This was evident when males were kept under food deprivation and also when they were re-fed. In the group of males supplied with carotenoids, food-restricted males probably incorporated less pigments into skin. Hence, independently of carotenoid intake, the ability of male boobies to incorporate pigments into the foot integument may be sensitive to nutritional state. Similarly, nutritional deprivation reduced plumage colour and carotenoid plasma concentration in male goldfinches (*Carduelis tritis*) that received a diet supplement of carotenoids (McGraw et al. 2005b). Some mechanisms of carotenoid utilization may explain the altered foot colour of

food-deprived birds. The absorption and transport of carotenoids can be sensitive to lipids and lipoproteins (Solomon and Bulux 1993) that are reduced during food deprivation. In addition, since carotenoids are potent antioxidants and immunostimulants (Blount et al. 2003; McGraw and Ardia 2003), food-restricted birds may have allocated more carotenoids to a depressed immune system or to reduce oxidative injury. In animals, prolonged fasting is known to affect the antioxidant capacity of the cell (Martensson 1986; Grattagliano et al. 2000), predisposing organisms to a reduced tolerance to oxidative injury.

In our experiment, variation of dietary carotenoids resulted in comparable changes in cell-mediated immune function and coloration, as reported in zebra finches (*Taeniopygia guttata*; Blount et al. 2003; McGraw and Ardia 2003). These results suggest that carotenoids serve as potent immune-enhancers and that carotenoid-pigmentation can reveal the immunological state of individuals. Overall, our results suggest that foot colour is a broad signal of current health and condition. Our experiment only manipulated phenotypic condition, but it is likely that the ability to handle stressful conditions has some genetic basis (Hamilton and Zuk 1982; David et al. 2000). This view is supported by a cross-fostering experiment which showed that blue-footed booby offspring condition correlates with the foot colour of the foster father, but also with the foot colour of the genetic father (Velando et al. 2005).

Our results also demonstrate that rapid change in male foot colour causes parallel variation in female investment. Previous experiments showed that females adjusted egg size or quality according to static male sexual traits (de Lope and Møller 1995; Limbourg et al. 2004), but as far as we know, this is the first study that demonstrates that females' investment can be influenced by a dynamic honest sexual signal. In our experiment, compared with control females, experimental females laid smaller second eggs when the foot colour of their mates became duller during the laying sequence.

In birds, egg size has been associated with the embryo's development, hatching success, size at hatching and fledging, and survival (review in Williams 1994). In the Nazca booby (*Sula granti*) the period between yolk completion and egg laying is approximately two days (Clifford and Anderson 2001). Since we manipulated the males three days before the second egg was laid, the reduction of second egg volume by experimental females was probably due to decreases of albumen mass and, to some extent, of the mass in the external yolk layers deposited. Experimental removal of the albumen content produces a strong decrease in hatching success and hatchling size (Finkler et al. 1998). In

the blue-footed booby, egg mass and volume correlated positively with hatching success and the mass of the chicks at hatching (D'Alba 2000). Furthermore, clutches in our experiment were early in the season, and previous studies show that females from natural early clutches lay relatively heavier second eggs, as control females in our experiment did (D'Alba 2000). By decreasing egg size, experimental females are probably facilitating brood reduction, and hence adjusting breeding investment in response to variation in male foot colour, a real-time indicator of condition.

This study suggests that blue-footed booby females perform a rapid fine-tuned adjustment of breeding investment using a dynamic condition-dependent sexual trait. Rapid changes in mate foot colour probably indicate a decrease in health or condition, and consequently, a reduced ability to invest in the brood (Velando et al. 2005), and/or low genetic quality (Hamilton and Zuk 1982; David et al. 2000). Adjustment of the investment in the egg in response to changes in the male's foot colour would therefore seem adaptive. Accordingly, females have synchronized their modification of breeding decisions in a short period of time with a highly dynamic signalling system. This study shows that, for a monogamous bird, mate evaluation and breeding decisions continue after pairing. In addition, the fact that females allocate resources to eggs based on the attractiveness of the mate highlights the importance of the social context in shaping a life history trait (Svensson and Sheldon 1998). We expect that condition-dependent dynamic traits, such as the colour in integuments, will be common and play a key role in monogamous animals with parental care.

Acknowledgments We thank Emma Cunningham, Diego Gil, Juan Freire, Dave Anderson, Kevin McGraw and Carol Vleck for valuable comments on the manuscript, Ana María Estrada Sánchez for helping during field work, Pablo Lago Velando for donating carotenoids and Glenn Furnier for improving the English. The study was supported by the Universidad Nacional Autónoma de México (IN230603). Logistic support was provided by the Armada de México, the staff from the Parque Nacional Isla Isabel and the fisherman from San Blas and Camichin. During the study, AV was supported with a grant from Universidade de Vigo and "Ramón y Cajal" fellowship from the Spanish Ministerio de Ciencia y Tecnología. The experiments performed comply with the current laws of Mexico, where the work was performed (permissions from SEMARNAT 01907 and 03687).

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