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Original Article

# Sex-specific phenotypic integration: endocrine profiles, coloration, and behavior in fledgling boobies

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The intensity of color expression in animals plays a key role in social environments as a mechanism to signal individual capacities in competitive contests. Selective pressures for resource competition differ at different stages of life and between sexes; therefore, coloration is expected to vary between juveniles and adults and between males and females. Exploring the covariance between coloration and other traits may help to understand the functional significance of color and the action of natural selection on multivariate phenotypes. Melanin-based plumage coloration was investigated in the masked booby *Sula dactylatra* in relation to melanin concentration, sex, hormone levels, and shy–bold behavior of chicks close to fledging. Darker brown boobies showed higher levels of both eumelanin and pheomelanin concentration and lower body mass. Males behaved bolder than females and showed on average 8% larger brown patches. Bolder females had smaller brown patches. Bolder individuals also had lower levels of circulating testosterone, but no differences in corticosterone levels were found. Stronger phenotypic integration was observed in females than males. Our study suggests that juvenile melanic coloration may reflect behavioral strategies by sex, endocrine profiles, and body mass indicating the convergence of different adaptive functions in a given phenotype, this being more evident in females. Direction of correlations differed from those predicted under the pleiotropic idea for color-related traits. These results suggest the possibility that juvenile plumage acts as a signaling system in a social context within the age class and suggest that plumage coloration may indicate different behavioral strategies.

*Key words*: **behavioral strategy, corticosterone, juvenile plumage, melanin pigment, personality, pleiotropy, Sula dactylatra, testosterone.**

## INTRODUCTION

Complex phenotypes can be viewed as integrated phenotypic traits reflecting genetic, developmental, or functional interactions ("phenotypic integration"; Pigliucci and Preston 2004). Coloration is one of the most diverse phenotypic traits in nature, and its variability is commonly associated with morph-specific phenotypic integration (e.g., Frey 2007; Forsman et al. 2008; McKinnon and Pierotti 2010; Santos and Cannatella 2011). Melanin pigments (eumelanin and pheomelanin) confer coloration on a broad range of vertebrate species (Bagnara et al. 1979; Hoekstra 2006; Hubbard et al. 2010). Although part of melanin-based phenotypes can be affected by environmental conditions, at least in birds (Griffith et al. 1999; Fitze and Richner 2002; Fargallo et al. 2007a, Catoni et al. 2009), it is recognized to have a strong genetic basis (Hubbard et al. 2010). Some of the genes involved in the expression of melanin-based coloration (mainly proopiomelanocortin [POMC] and melanocortin receptors) are also responsible for the regulation of other functions, such as immunity, energy homeostasis, food intake, and stress response or behavior (Mundy 2005; Cone 2006; Ducrest et al. 2008); thus, potential pleiotropic effects could account for

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the covariation of melanic coloration with other phenotypic traits (Gompel and Carroll 2003; Ducrest et al. 2008; Kim et al. 2013).

Melanin-based coloration varies from pale gray to black and from yellow to brown, including reddish colorations in different combinations of eumelanin and pheomelanin pigments (McGraw 2006). Although it is not really understood how the final coloration derives from both pigments, many authors have assumed for birds the rule that black/gray is eumelanin dependent; and yellow, brown, and reddish are pheomelanin dependent, considering the most prevalent pigment in each case (e.g., Delhey et al. 2010; Galván et al. 2011; Meunier et al, 2011; Roulin et al. 2011). In general, the degree of dark melanic coloration in animals is positively correlated with levels of dominance and aggressiveness via testosterone effects ("T-regulation hypothesis"; Jawor and Breitwisch 2003; Bókony et al. 2008), as T favors aggressive behavior (Wingfield et al. 1990; Alonso-Álvarez and Velando 2001) and regulates melanin production (Wilson 1983; Evans et al. 2000; Fargallo et al. 2007b). Besides considering the potential environmental causes promoting the intercorrelation in the expression of different characters, another proposed possibility is that covariation among these different traits may occur through the pleiotropic effect of the genes involved in melanogenesis (reviewed by Ducrest et al. 2008). It is believed that melanogenesis is controlled in part by the melanocortin system that refers to a set of hormonal, neuropeptidergic, and paracrine signaling pathways that are defined by components that include the 5 G-protein-coupled melanocortin receptors (MC1R to MC5R), peptide agonists derived from the POMC preprohormone precursor (α-, β-, γ-melanin stimulating hormones [MSHs], and the adrenocorticotropin hormone [ACTH]) and the endogenous antagonists (agouti and agouti-signaling protein; Cone 2006). This signaling system regulates a remarkably diverse array of physiological functions including pigmentation, adrenocortical steroidogenesis, energy homeostasis, and exocrine gland secretion (Cone 2006). High levels or high MC1R agonist activity of the melanocortins (mainly α-, β-MSH, and ACTH) are predicted to increase eumelanogenesis and decrease pheomelanogenesis (Ducrest et al. 2008; Hubbard et al. 2010; Roulin and Ducrest 2011). Through binding to the other 4 receptors, melanocortins regulate physiological functions, including hypothalamic–pituitary–adrenal (HPA) stress response and steroidogenesis (MC2R), food intake (MC3R and MC4R), and aggressiveness (MC5R; Cone 2006; Morgan and Cone 2006). Consequently, these pleiotropic effects predict associations between these behavioral and physiological traits and melanin-based coloration (Ducrest et al. 2008).

The finding that regulators of the melanocortin system can pleiotropically affect the expression of suites of correlated phenotypic traits has important implications with regard to the existence of behavioral strategies. Animals adopt different behavioral strategies to cope with environmental challenges (Koolhaas et al. 1999, 2010; Sih et al. 2004; Korte et al. 2005; Réale et al. 2007). The set of behavioral and physiological responses, which are consistent over time and across contexts, has been defined in many ways, for example, coping styles, temperaments, behavioral tendencies, strategies, syndromes, and axes (e.g., Sih et al. 2004; Réale et al. 2007); here, we will use the term "behavioral strategy" to refer to this. One axis of variation is the shy–bold gradient (Wilson et al. 1994; Budaev 1999; Wilson and Godin 2009; Carter et al. 2010). In one extreme of this gradient are individuals usually called bold, proactive, "hawks," or fast explorers characterized by performing more aggressive and daring behavior, higher leaning toward taking risks in challenging situations, and faster and more superficial territory explorations which in general fit a so-called fight–flight behavioral strategy. In the opposite extreme are individuals called shy, reactive, "doves," or slow explorers tending to behave in a more cooperative and timid way, with a lower propensity to take risks in challenging situations and exploring territories in a slow and thorough way, fitting a "freeze-hide" strategy (Koolhaas et al. 1999; Veenema et al. 2003; Dingemanse et al. 2004; Korte et al. 2005). There is clear evidence for the genetic basis of these behavioral traits (de Boer et al. 2003; Drent et al. 2003; Ariyomo et al. 2013), whose variation in a population might be maintained through heterogeneity in selection pressures that favor each strategy at different spatial or temporal scales (Maynard Smith 1982; Dall et al. 2004).

Behavioral strategies have been found to co-vary with coloration and physiological traits. This integration at different levels of organization may reflect proximate causal mechanisms inducing the response to challenging situations. Broadly, bolder or more proactive individuals show relatively lower reactivity of the HPA axis (involving lower plasma corticosterone [CORT] levels) in response to stressful situations, and the contrary is observed for shyer or more reactive individuals (Koolhaas et al. 1999; Korte et al. 2005). Parallelisms maintained between behavioral and neuroendocrine traits are believed to be due to genetic covariation and co-selection (Price and Langen 1992; Marchetti and Drent 2000; Drent et al. 2003). Interestingly, phenotypic integration may be different in males and females, due to sex-specific selection on life-history profiles and underlying physiological systems (Stoehr and Kokko 2006; Ketterson et al. 2009)

Sex and age, among other factors, are responsible for within-population heterogeneities in behavioral strategies (Blanckenhorn 2005; Réale et al. 2007). Sexual dimorphism in size, coloration, and behavior can be the result of divergent adaptive strategies in response to selection pressures that differ between the sexes (Andersson 1994; Fairbairn 1997; Blanckenhorn 2005). Selection pressures can differ at different life stages; therefore, sexual dimorphism is also expected to vary with age class; furthermore, selection acting on the growth of males and females will result in changes in the final adult sexual dimorphism (see Badyaev 2002). These age-dependent pressures have served to explain, for example, why sexes of dimorphic species are similar during early development (Badyaev 2002).

Melanic coloration is expressed in adult and juvenile phenotypes. Despite this, virtually all the research has focused on sexually mature individuals and very little is known on the functional significance of juvenile coloration. Juvenile plumages are markedly different from adult plumages in many bird species (Senar 2006; Moreno and Soler 2011). It has been considered that the main function of juvenile plumages in a social context is to signal subordination to and reduce aggression from adults. It is still unclear whether juvenile plumages can act as a real signal of status (reviewed by Senar 2006) because this type of signal is originally believed to work both within and between age and sex classes (Rohwer 1975). Furthermore, it is unknown whether juvenile coloration is subject to the same selective pressures that promote adult color variation. It is also poorly understood how and why juvenile plumage varies. In some of the few studies on this subject, individual juvenile coloration has been considered a signaling system addressed to parents, which influence allocation of parental resources (Lyon et al. 1994; Penteriani et al. 2007; Galván et al. 2008; Ligon and Hill 2010). However, juvenile plumage can also signal dominance and individual competitive capacities within the juvenile age class (Jones 1990; Vergara and Fargallo 2008; Vergara et al. 2010; Tringali and

Bowman 2012). If so, juvenile plumage coloration may be a channel to communicate behavioral strategies and phenotypic profiles.

In this study, we investigate the relationship between melanin-based coloration, sex, hormones, and behavior of masked booby *Sula dactylatra* chicks close to fledging (hereafter fledglings). This species shows low variability in adult coloration (sexual monomorphism) but a distinctive variation in juvenile plumage during the first 3 years of life (Nelson 1978). We first determined melanin composition and concentration of the brown coloration of juvenile plumage and the relationship with fledgling plumage variability. Second, we examined potential sex differences in fledglings' body size, plumage color, and hormone levels (plasma corticosterone [CORT] and testosterone [T] concentration). Third, fledglings were subjected to a behavioral test in which flight initiation distance (FID) and aggressiveness after a challenge were recorded as an estimation of shy–bold behavioral axis. We investigated whether this behavior was related to coloration and hormone levels of males and females. We predict that if pleiotropic effects exist in the direction proposed by Ducrest et al. (2008), individuals producing more eumelanin should be darker (lower lightness values), bolder, less heavy, and should show lower levels of CORT and higher levels of T. Finally, we tested whether phenotypic integration differs between sexes by means of structural equation modeling (SEM).

## MATERIAL AND METHODS

### Study species

The masked booby is the largest booby species exhibiting a strong reversed sexual size dimorphism (females being 14% heavier) and sexual chromatic monomorphism (Nelson 1978). Although masked boobies lay 2 eggs, they are obligate siblicides rearing only a single chick (Nelson 1978; Weimerskirch et al. 2009). About 60 days after hatching, chicks are left unguarded and fed 1.4 times a day on average by adults (Nelson 1978). In contrast to the mainly black and white adults, fledglings and yearlings show a varying brown-white coloration on the crown, forehead, back of the neck, sides of the neck, nape, ear coverts, back, and rump (Figure 1). Tail, scapulars, and lesser and median wing coverts are brown with lighter or even white edgings (Figure 1). Plumage replacement in fledglings begins some months after they can fly. The head starts first turning slightly speckled brown 9 months later and pure white after 14–15 months. Back and wing coverts appear slightly flecked brown after 14 months and almost white after 17 months. The full adult plumage is reached 3–4 months before age 3 (see Nelson 2005).

### Study area and behavioral test

The study was conducted during November 2010 in the masked booby colony of about 2150 breeding pairs from Muertos Island, located at the National Park Arrecife Alacranes (Gulf of Mexico, 22°25′11″N, 89°42′56″W). At the end of November, 84% of the pairs were found in the chick rearing-fledging phase. For this study, only fledglings ($n$ = 57) of about 90–100 days old were included (age estimation based on wing length; Nelson 1978). During the fledging period, unguarded fledglings stay at the beach waiting to be fed by their parents. Usually fledglings were observed aggregated in groups, and solitary fledglings were rarely observed in the beach (Authors, unpublished data). The experiment and animal handling were done in the morning (08:00–11:00 h) and in the afternoon (16:00–20:00 h) avoiding warmest hours of the day. FID was estimated following Martín et al. (2006). Briefly, when an unguarded fledgling was discovered at the beach, a researcher approached it by walking directly toward the focal bird, whereas

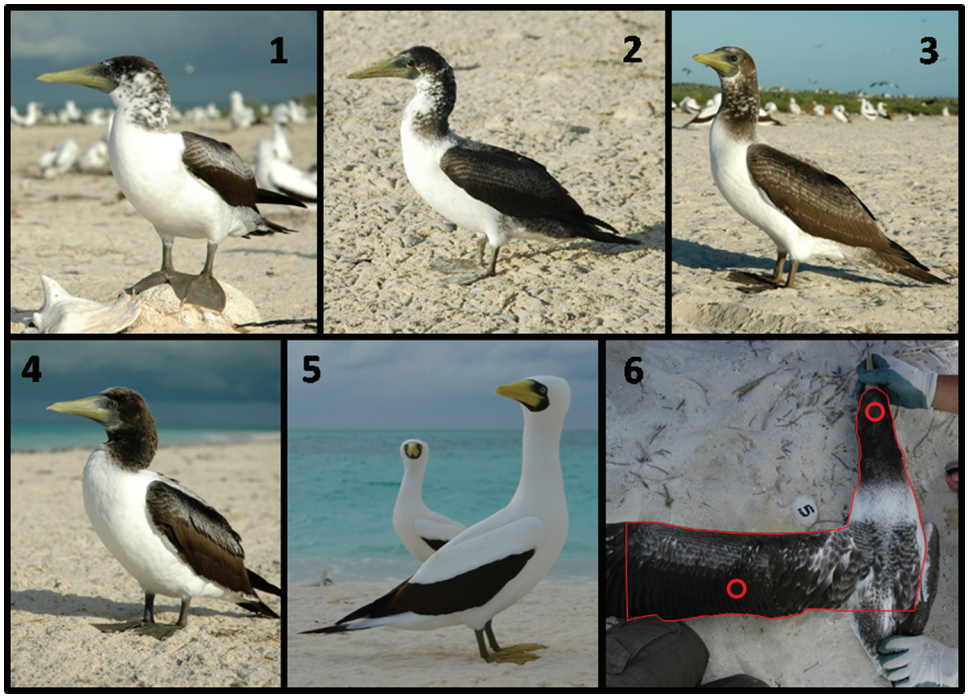


**Figure 1**
Phenotypic variation of melanin-based coloration of masked boobies close to fledging (90–100 days of age) at Muertos Island, Gulf of Mexico. A brown-white coloration gradient is represented from whiter (1) to browner (4) individuals. Panel 5 shows 2 adult masked boobies. Panel 6 shows the area where the back brown patch (percentage) was measured. Circles in panel 6 indicate the points where brown chroma and lightness were measured.

an observer placed 25 m away estimated the distance at which the bird first moved away (straight line measured to the nearest 0.5 m) from the approaching researcher. To avoid confounding effects, the same researcher (A.V.) wearing the same clothing performed all approaches in a similar way, walking at the same medium speed. FID was estimated as the mean value estimated by A.V. and the observer. All approaches started 30 m from the bird. This distance was established after some preliminary trials in which the behavior of fledglings was examined. At this distance, the animals behaved normally, without displacement activities (preening, scratching, etc.) or flight initiation. A.V. made a direct approach from the shore toward the booby by walking at ~90–100 m/min and looking straight at focal bird (Martín et al. 2006). After moving and before flying away, the fledglings were captured by hand. Because the proximity to conspecifics may affect individual FID (e.g., Fernández-Juricic et al. 2002, but see Martín et al. 2006), only individuals located within fledging aggregations were captured. Solitary fledglings were not included in our study.

Captured birds were taken to the shore (30–50 m distant from capture site) to carry out a restraint test in order to evaluate individual aggressive behavior. For this test, A.V. held the bird (preventing wing movements) and presented it to the same researcher (J.A.F.) always wearing the same clothing and situated 1 m in front of the bird. J.A.F. slowly offered and took away 3 times (10 s between each) a hand covered by a gray woolen glove. The number of pecks was defined as the number of times that the bird pecked the hand (ranging from 0 to 3). The intensity of pecking was also estimated as: 0 = no pecks, 1 = pecking and releasing the glove, 2 = pecking and keeping hold the glove when taking the hand away, and 3 = pecking, keeping, and twisting the glove. Although little within-individual variation was observed in the way they pecked the glove, the value assigned to each individual was the highest intensity of pecking observed in any of the 3 hand trials, which is similar to the average as there was little within-individual variation. The tests were always performed in the same way by placing the bird with its back to the sun. A similar test was carried out in chinstrap penguins *Pygoscelis Antarctica*, and the number of pecks to humans was considered to be a good index of aggressiveness (Viñuela et al. 1995). The number of pecks and pecking intensity was positively correlated ($r_s = 0.70$, $P < 0.001$, $n = 57$), and the FID tended to be, but not significantly so, positively correlated with number of pecks ($r_s = 0.23$, $P = 0.086$, $n = 57$) and pecking intensity ($r_s = 0.22$, $P = 0.100$, $n = 57$). For this reason, the 3 variables were combined in a principal components analysis that resulted in only 1 axis (PC1) explaining 61% of the variance (eigenvalue = 1.83; factor loadings for number of pecks = 0.89, pecking intensity = 0.89, and FID = 0.49). PC1 did not differ from a normal distribution (Kolmogorov–Smirnov, $d = 0.14$, $P > 0.20$). The resultant PC1 was considered an index of shy–bold behavior measured in 57 fledglings (24 females and 33 males). Positive values in the PC1 represent individuals displaying bolder behavior and negative values represent shyer behavior.

## Hormone measurement and sex determination

When the behavioral test was finished, 1 mL of blood was immediately collected from the brachial vein with a 1-mL heparinized syringe. Time from capture to bleeding was recorded and never exceeded 11 min (mean ± SD = 4.1 ± 1.5; range = 1.5–10.4 min). Samples were kept cool for the rest of the day (maximum 4 h) and subsequently centrifuged at 10 000 r.p.m. for 10 min. The separated fractions of plasma and blood cells (pellet) were stored in microtubes at −80°C in a liquid-nitrogen tanks for later analyses in the laboratory. Concentrations of T and CORT were determined by 2 different EIA kits highly specific for each hormone (DRG Labs, Germany). According to the manufacturers, cross-reactivity of the T antibody was less than 1% for all hormones tested, and cross reactivity of the CORT antibody was less than 1% for all hormones tested except for 11-deoxycorticosterone, which was 2.4%. Assays were performed according to manufacturer's instructions. In an initial assay, plasma samples were found to contain less T than the lowest standard of the kit. Therefore, samples were concentrated by extracting T with a volume of 10× diethyl ether from plasma samples and resuspending in a double volume of ultrapure water. In contrast, in an initial assay, plasma samples were found to contain more CORT than the highest standard; hence, samples were diluted to the half with the ultrapure water. Samples were analyzed in duplicate with respect to a standard curve, and a set of identical internal controls was run in each assay. The interassay variation, after correction by means of linear regressions of these controls, was 0.18% for T and 5.60% for CORT. The intra-assay coefficient of variation for T was 7.70% and 12.12% for CORT. Samples were randomized across plates with respect to sex and date and time of capture.

For sex determination, DNA was extracted from blood pellets using the PUREGENE protocol (Gentra Systems, Minneapolis, MN). Around 20 ng of DNA solution from each chick was used in a polymerase chain reaction to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (Griffiths et al., 1998).

## Body and color measurements

Booby fledglings were weighed with a spring balance (±5 g). Flattened wing chord and wing length were measured using a metallic ruler with a stop (±1 mm). Tarsus and bill length were measured using a caliper (±0.1 mm). Also in a subsample of 15 birds, 3 major covert feathers were randomly collected from the wing. Removal of feathers was performed by pulling them near the base. Feathers were kept in plastic bags until melanin analyses were performed.

Color in head and major wing coverts was measured by taking 3 measures with a spectrophotometer (Minolta CM-2600d) in each body part. We used the L*a*b* color space, where L* indicates lightness, and a* and b* indicate the chromaticity coordinates. The saturation of the color given by the coordinates a* and b* increases as the absolute values of a* and b* increase (Montgomerie 2006). Only lightness and a* axis (coordinate varying from red to green and including brown) were used as lightness and chroma color components.

The area of brown patches on the body was measured by digitally photographing all captured birds (camera Nikon D70, objective: 18–70 mm AFS Nikkor DX). A picture from the back side was taken while the booby was held with the neck and wing stretched. A second profile picture from the stretched neck was taken. All pictures were taken when the bird was under a sunshade. The images were imported into the ImageJ 1.33u program developed at the US National Institutes of Health, US (http://rsb.info.nih.gov/ij/Java1.3.1_03). The brown patch is easily distinguishable from the white background of the booby plumage; hence, the brown patch was outlined, and its area was determined as the number of pixels occupied using the "analyze measure" function. Brown patch area was calculated relative to the area of the photographed bird body (back and neck profile) and defined as the percentage of brown covering the photographed area from neck, back, and

wing. To estimate measurement repeatability, the brown patch in pictures from 15 randomly selected individuals was measured again 1 month later. Repeatability of the measurements was high ($r = 0.95$).

Feather coloration variables were combined in a principal components analysis (Table 1) that resulted in only one PC1 explaining 57% of the variance (eigenvalue = 2.3) and represented a darkness gradient of head and wing feather coloration varying from high values of chroma and low values of lightness in the positive extreme to low values of chroma and high values of lightness in the negative extreme (factor loadings: head lightness = −0.80, head chroma = 0.72, wing lightness = −0.79, wing chroma = 0.71).

## Melanin composition and concentration in feathers

Melanin composition and concentration were measured in 15 major covert feathers belonging to 15 different individuals analyzed. Pheomelanin and eumelanin concentration was estimated as described in Wakamatsu et al. (2002) and Ito et al. (2011). Briefly, 4–7 mg of feather from the middle part were cut and homogenized with a glass homogenizer in water (10 mg/mL). Sample homogenate (100 µL) was taken in a 10 mL screw (Teflon)-capped conical glass test tube to which 375 µL 1 mol/L $K_2CO_3$ and 25 µL 30% $H_2O_2$ (final concentration: 1.5%) were added. The mixture was mixed vigorously at 25°C ± 1°C for 20 h. The residual $H_2O_2$ was decomposed by adding 50 µL of 10% $Na_2SO_3$, and the mixture was then acidified with 140 µL of 6 mol/L HCl. After vortex-mixing, the reaction mixture was transferred to 1.5-mL Eppendorf tube and centrifuged at 4 000 × $g$ for 1 min, and an aliquot (80 µL) of the supernatant was directly injected into the HPLC system with UV-VIS detector (269 nm). The amount of eumelanin can be obtained by multiplying the amount of pyrrole-2,3,5-tricarboxylic acid by a conversion factor of 25. To determine pheomelanin concentration 100 µL of a feather sample homogenate was hydrolyzed with 500 µL 57% HI and 30 µL 30% $H_3PO_2$ at 130°C for 20 h, after which the mixture was cooled. The product (4-amino-3-hydroxyphenylalanine; 4-AHP) was analyzed using high-performance liquid chromatography with electrochemical detection (Wakamatsu et al. 2002). The amount of pheomelanin can be obtained by multiplying the amount of 4-AHP by a conversion factor of 9.

## Statistical procedures

General linear models (GLM) in SAS software 9.2 (SAS Institute Inc., Cary, NC) were used for the analyses. Models for feather coloration and brown patch size as response variables included body measurements (body mass, bill, tarsus, wing, and wing chord lengths) as covariates and sex as a fixed factor. Models for plasma levels of CORT and T included body measurements, feather coloration, and brown patch size as covariates and sex as a fixed factor. Models for shy–bold behavior included body measurements, feather coloration, brown patch size, and hormone levels as covariates and sex as a fixed factor. In all GLMs, the effect of explanatory variables were fitted following forward stepwise procedure (e.g., Emerson and Kolm 2005), testing the significance of each variable one by one, testing the addition of each variable, and choosing the variable that resulted in the largest increase in the fit of the model according to Akaike's Information Criterion (AIC) and repeating this process until none improved the model. Interactions between covariates and fixed factors were also explored in all models. Plasma CORT concentration was log transformed to obtain a normal distribution (Kolmogorov–Smirnov, $W = 0.1$, $P > 0.2$). Residuals from all models showed normal distributions (Kolmogorov–Smirnov, all $P > 0.05$). All tests were 2-tailed with alpha set at 0.05.

To investigate whether phenotypic traits exhibited sex-specific patterns of covariance, SEM was applied. Shy–bold behavior was modeled as a "latent variable," an unobserved variable indirectly measured by the 3 behavioral tests (see Dingemanse et al. 2010; Edelaar et al. 2012). To keep the number of variables low enough to perform SEM with our sample size, independent variables with significant covariance with shy–bold behavior were selected based on a priori information (i.e., brown patch and T, see Results). The inclusion of body mass or CORT in SEMs did not significantly improve the models, and all path parameters of these variables were not statistically different from zero. Our data meet the assumption of nonrandom expectation (Bartlett's test of sphericity; $\chi^2 = 54.46$, $P < 0.001$), and the Kaiser–Meyer–Olkin index was greater than 0.5 (0.57) allowing multivariate comparisons.

Multigroup analysis was used for comparisons of path structure between sexes (Grace 2006; Arbuckle 2010). Dissimilarity of behavioral matrix between sexes was first estimated by comparing a model with similar behavioral structure with another that allow sex-specific covariance between latent variable shy–bold behavior and behavior measurements. Second, to evaluate whether the structure of path parameters (behavior, color, and hormone concentration) differs between sexes, a model with similar path parameters was compared with a model with sex-specific path parameters (Grace 2006; Arbuckle 2010). In order to evaluate the models, the AIC and $C_{min}$ were used. AIC values were considered to select the most parsimonious model (i.e., the model with lower discrepancies ($\hat{C}$) and complexity). For each nested pair of models (as described above: sex-specific behavior and sex-specific path structure), $C_{min}$, the minimum value of the discrepancy ($\hat{C}$) was calculated by maximum likelihood estimation, which has an approximate chi-square distribution with degrees of freedom equal to the difference between the degrees of freedom of the competing models (Arbuckle 2010). The chi-square goodness of fit of our final model represents an overall measure of how much the implied covariances differ from the sampled covariances (i.e., smaller chi square indicates greater similarity between the implied and sampled covariances; Arbuckle 2010), was also computed.

**Table 1**

**Behavioral strategy within the shy–bold axis of masked booby fledglings**

| Term | Estimate | $F$ | $P$ |
|---|---|---|---|
| Testosterone | −12.9129 | 8.8 | 0.005 |
| Sex | 5.4440 | 5.8 | 0.020 |
| Brown patch size | 0.0259 | 1.5 | 0.244 |
| Sex × brown patch size | −0.0936 | 7.1 | 0.011 |

The final GLM model is presented showing the effect of plasma T concentration, sex, and brown patch size on behavior (model $F_{5,51} = 4.6$, $P = 0.003$, $r^2 = 0.26$, AIC = 50.34).

# RESULTS

## Melanin composition and concentration

Brown coloration in feathers was mainly composed by eumelanin (eumelanin = 11 220.2 ± 3106.6 ng of pigment/mg of feather; pheomelanin = 134.8 ± 40.6 ng/mg) and a mean 85.5 ± 19.1 eumelanin–pheomelanin ratio (E/P). Feathers containing more

eumelanin also had more pheomelanin concentration ($r$ = 0.70, $F_{1,13}$ = 12.1, $P$ = 0.004, $n$ = 15). Lightness was negatively correlated with eumelanin ($r$ = −0.73, $F_{1,13}$ = 15.1, $P$ = 0.002, $n$ = 15) and pheomelanin ($r$ = −0.57, $F_{1,13}$ = 6.2, $P$ = 0.027, $n$ = 15) concentrations but not with E/P ($P$ = 0.37). Chroma was not correlated with melanin pigments or E/P (all $P$ > 0.28). Brown patch size was not significantly correlated with feather coloration ($r$ = 0.17, $F_{1,55}$ = 1.7, $P$ = 0.194, $n$ = 57). However, brown patch size was negatively correlated with pheomelanin concentration ($r$ = −0.70, $F_{1,13}$ = 12.1, $P$ = 0.004, $n$ = 15; Figure 2). The dot inside the circle in Figure 2 represents an outlier observation for pheomelanin concentration (Grubb's Test Statistic = 2.8, $P$ = 0.023). When excluding that value from the regression model, both variables were still significantly correlated ($r$ = 0.73, $F_{1,12}$ = 15.0, $P$ = 0.002, $n$ = 14). Brown patch size was not significantly correlated with eumelanin concentration ($P$ = 0.14) and only marginally correlated with E/P ($r$ = 0.47, $P$ = 0.076, $n$ = 15).

## Sex-related body measurements, coloration, and circulating hormones

At the age of 90–100 days, masked boobies did not show significant sexual differences in wing length (GLM, $P$ = 0.33; males (m) = 400.5 ± 2.6 mm, females (f) = 404.5 ± 3.1 mm). Controlling for wing length, fledgling females were heavier and had longer bills, tarsus, and wing chords than males (GLM, all $P$ < 0.001; weight: m = 1484.5 ± 23.3, f = 1750.2 ± 27.4; bill length: m = 97.9 ± 0.4, f = 100.3 ± 0.5; tarsus length: m = 55.0 ± 0.2, f = 56.5 ± 0.3; wing chord: m = 202.0 ± 0.8, f = 209.7 ± 1.0).

Feather coloration (PC1) differed significantly between sexes (GLM, $F_{1,55}$ = 9.7, $P$ = 0.003, $n$ = 57), males being darker and with higher chroma values than females. However, when body mass was included in the model, sex effect was not statistically significant (GLM, sex: $F_{1,53}$ = 0.8, $P$ = 0.384; body mass: $F_{1,53}$ = 5.3, $P$ = 0.026, $n$ = 57). The interaction sex × body mass was not significant (GLM, $F_{1,53}$ = 0.9, $P$ = 0.339, $n$ = 57). Removing the interaction from the model, feather coloration was only explained by body mass (GLM, $F_{1,55}$ = 15.0, $P$ < 0.001, $n$ = 57). Darker individuals showed lower body mass irrespective of sex (Figure 3). The dot inside the circle in Figure 3 represents an outlier observation for feather coloration (Grubb's Test Statistic = 4.3, $P$ < 0.001). After excluding that value from the model, body mass and feather coloration were still significantly correlated (GLM, $F_{1,52}$ = 9.8, $P$ = 0.003). Differences by sex and the interaction sex × body mass were not significant (GLM, both $P$ > 0.2, $n$ = 56). Morphological measurements (bill, wing, wing chord, and tarsus length) and their interaction with sex were not significant (GLM, all $P$ > 0.22). Males showed larger brown patches than females (males = 67.7 ± 1.2%, females = 60.1 ± 1.4%; GLM, $F_{1,55}$ = 17.3, $P$ = 0.002, $n$ = 57). Brown patch size was not correlated with any morphological measurements (bill, wing, wing chord, and tarsus lengths) or with body mass (GLM, all $P$ > 0.16).

Circulating CORT concentration was positively correlated with the time from capturing to bleeding ($r$ = 0.27, $F_{1,55}$ = 4.6, $P$ = 0.036, $R^2$ = 0.08, $n$ = 57) and with the squared term of time ($r$ = 0.42, $F_{2,54}$ = 5.6, $R^2$ = 0.17; time $P$ = 0.005, time$^2$ $P$ = 0.016), indicating that CORT levels significantly fit to a curvilinear function. Thus, the terms "time" and "time$^2$" were included in the analyses. T and CORT plasma concentrations were not correlated ($P$ = 0.955). Neither CORT nor T circulating levels were significantly correlated with body measurements (bill, wing, wing chord, and tarsus lengths), body mass, coloration, sex, and interactions with sex (GLM, all $P$ > 0.19).

## Sex-related behavioral strategies

Males showed bolder behavior than females (Table 1; Figure 4a). This shy–bold behavior axis was significantly correlated with the brown patch size but in a different way for males and females (interaction sex × brown patch size; Table 1). Females with a smaller brown patch behaved in a bolder way than those showing more brown-colored feathers, whereas bolder males had slightly larger brown patch (Figure 4b). Behavior was also significantly correlated with plasma T levels (Table 1). Individuals from both sexes having lower plasma T concentration behaved in a bolder way than individuals with higher T levels (Figure 4c). The sex × T interaction was not significant (GLM, $F_{1,51}$ = 2.1, $P$ = 0.15, $n$ = 57). Feather coloration, CORT concentration, and body

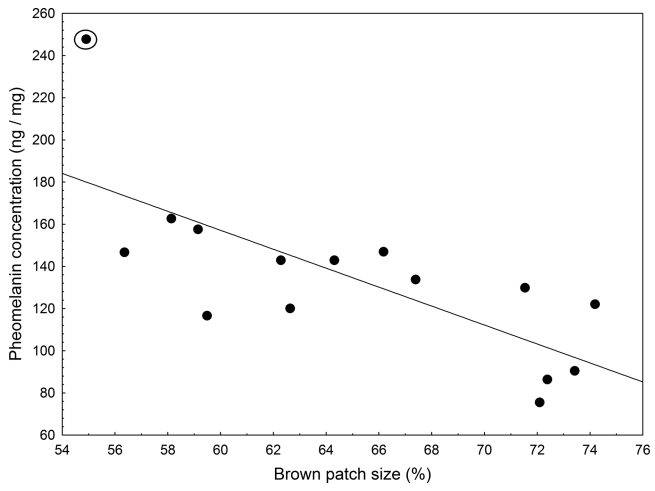


**Figure 2**
Relationship between pheomelanin concentration in feathers and the extent of brown patch on the body of masked booby fledglings. The dot inside a circle represents an outlier observation (see text).

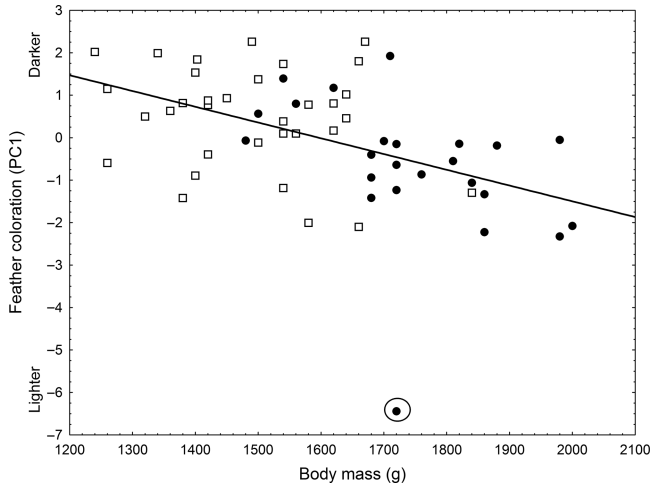


**Figure 3**
Relationship between feather coloration (PC1 includes head and wing lightness and chroma) and body mass in masked booby fledglings. Black dots represent females and white squares represent males. The dot inside a circle represents an outlier observation (see text).

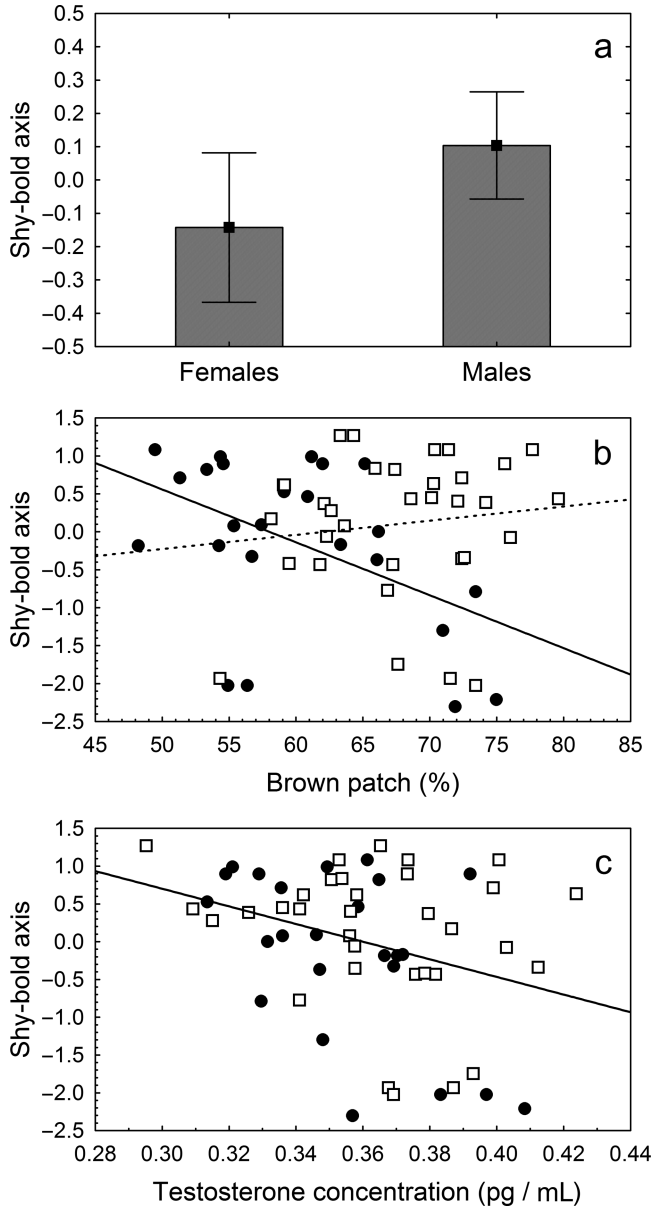


**Figure 4**
(a) Sexual differences in shy–bold behavior. (b) Sexual differences in the relationship between behavior and brown patch size in masked booby fledglings. Black dots and the continuous line represent females and white squares and the dotted line represent males. (c) Relationship between behavior and circulating testosterone concentration. Black dots represent females and white squares represent males. Black dots and the continuous line represent females and white squares and the dotted line represent males.

measurements were unrelated to fledglings' shy–bold behavior (all $P > 0.2$).

## Structural equation modeling

Overall, the SEM model that best explained the data was one where the structure of behavioral strategies did not differ between sexes, but with sex-specific covariance among behavior, color, and T (Figure 5; AIC = 47.54; $\chi^2$ = 13.54, degrees of freedom [df] = 12, $P$ = 0.51). Thus, the model with sex-specific covariance in the relationships between latent variable shy–bold behavior did not improve the model assuming similar covariance between sexes (ΔAIC = 1.28; $C_{min}$= 8.72, df = 4, $P$ = 0.12). Nevertheless, the model with similar structure in shy–bold behavior between sexes was improved by sex-specific covariance structure among behavior, color, and T (ΔAIC = −3.64; $C_{min}$= 7.64, df = 3, $P$ = 0.022). Thus, behavior was significantly correlated with brown patch size and T in females but not in males (Figure 5).

# DISCUSSION

## Melanin-based coloration

Although it is accepted that brown coloration can be produced by both eumelanin and pheomelanin as the prevalent pigment (Jawor and Breitwisch 2003; McGraw 2006; Ito and Wakamatsu 2008), it is frequently assumed that gray and black colorations are mainly composed of eumelanins, whereas yellowish, reddish, chestnut, and brown colorations are mainly composed of pheomelanin (e.g., Delhey et al. 2010; Galván et al. 2011; Roulin et al. 2011, but see Ducrest et al. 2008; Roulin and Ducrest 2011). This "melanin pigment color rule" led to talk about pheomelanic and eumelanic color, traits, or individuals (e.g., Galván et al. 2011; Roulin and Ducrest 2011) and to the assumption that the main pigment responsible for feather coloration can be determined by simple observation (Galván et al. 2011) without performing full pigment analyses. In addition, Ducrest et al. (2008) proposed that color darkening is based on the increase of eumelanic pigmentation (brown or black) by triggering eumelanogenesis at the expense of reducing or blocking pheomelanogenesis (Ducrest et al. 2008; Roulin and Ducrest 2011). Our study, however, reveals that 1) feather brown coloration is mainly composed of eumelanin, 2) feather lightness was negatively correlated with both eumelanin and pheomelanin concentrations, and 3) the degree of brown body melanization (brown patch size) was negatively correlated with the minority pigment pheomelanin but not with the most abundant eumelanin. Our study, thus, clearly indicates that the melanin pigment coloration rule is not a general rule (see also McGraw 2006; Gasparini et al. 2009) and that the synthesis of a given pigment does not reduce or block the synthesis of the other.

## Sex-related size, coloration, and hormone levels

Sexual size dimorphism in the masked booby is already observed at the age of 90–100 days. Except for wing length, females have longer bills, tarsi, and wing chords, and are heavier than males. Also, our results showed that male and female masked boobies adopt different coloration strategies during early life. Differences were found in both the brown patch size and feather coloration, with males exhibiting a larger proportion of body surface covered by darker brown coloration than females. Interestingly, sexual differences in brown coloration were depended on body mass in such a way that the effect of sex was not significant when body mass was included in the model. This result suggests that sex-related color variation can be mainly determined by sex-related strategies of body mass gain, which partly agrees with the pleiotropic effect predicted for coloration and body mass (food intake) patterns (Ducrest et al. 2008). According to this prediction, higher levels or activity of melanocortins (mainly α-MSH and also β-MSH and ACTH) promote the darkening of coloration by binding MCR1 in the melanocytes, hence increasing the production of eumelanin. In addition, a high activity of these melanocortin hormones promotes a reduction of food intake by binding to MCR3 and MCR4 receptors placed in the central nervous system and adipose tissue (Ducrest et al. 2008),

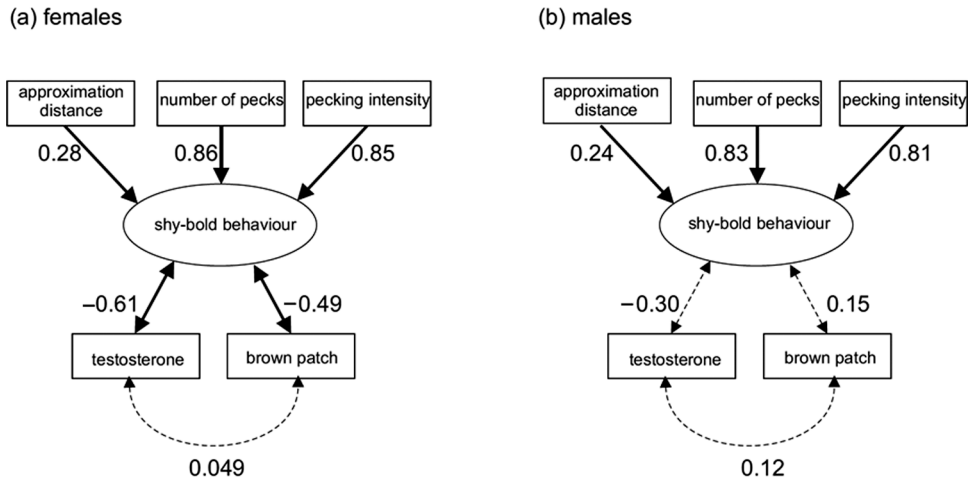


**Figure 5**
The best supported structural equation model of phenotypic integration between behavioral axis (a latent variable indirectly measured by 3 behavioral tests), testosterone levels, and melanin-based color (brown patch size) for (a) female and (b) male masked booby fledglings. Solid and dotted arrows represent significant and nonsignificant relationships, respectively ($\alpha = 0.05$). Numbers in the arrows are standardized effects underlying phenotypic structure.

although there is a great variation among species in the presence of the different MCRs in different tissues and in the expression of different MCRs in the adipose tissue (Cone 2006; Yabuuchi et al. 2010). Our results agree with the hypothesis as darker individuals (higher values in the PC1) showed lower body masses. Interestingly, feathers showing lower values of lightness also had both higher eumelanin and pheomelanin concentrations, suggesting that inter-individual differences in color-related body mass are not associated with a higher production of eumelanin with respect to pheomelanin but with a higher melanogenesis in general.

No differences between males and females were observed in CORT and T plasma concentrations. These results concur with those previously observed in other bird species at similar ages either for CORT (see Blas et al. 2006 and references therein) or T (Fargallo et al. 2007b and references therein; López-Rull et al. 2011).

## Behavioral strategy

Previous studies have suggested that animals, including seabirds, do not actually respond to the presence of a human as a predator but respond to the approach of a large object, which would be similar to the approach of a predator as they respond in a similar escaping way (see Martín et al. 2006 and references therein). It is not clear how exactly escape, aggressiveness, and boldness are interrelated (Korte et al. 2005; Koolhaas et al. 2010). It has been found, for example, that fish populations living in sites with high risks of predation show higher boldness and faster escape response than those living in safer sites (reviewed in Réale et al. 2007). In addition, more aggressive great tits *Parus major* were also more dominant, fast exploring, and bolder (Verbeek et al. 1996; van Oers et al. 2011). Also hypoactive (reactive) chaffinches *Fringilla coelebs* were more likely to freeze in a risky situation (predator presence), whereas more hyperactive (proactive) individuals are more prone to escape (Quinn and Cresswell 2005). Furthermore, in rodents, bolder and aggressive individuals have been observed to adopt a "flight" behavioral strategy when exposed to large dominant conspecifics, whereas shyer and nonaggressive individuals show freezing (de Boer et al. 2003 Korte et al. 2005). For this reason, considering the results obtained from our behavioral test (PC1), we assumed that more aggressive (higher number and intensity of pecks) individuals flying sooner (longer FID) in response to a potential predator approaching are also individuals exhibiting a bolder behavioral strategy. Repeatability estimations are, however, needed to know whether this behavioral assay reflects stable attributes of the individuals (Bell et al. 2009).

## Sexually related behavior

Considering the shy–bold behavioral axis, female booby chicks were more prone to develop the former strategy and males the later strategy. In other words, males behaved in a bolder way, that is, they escaped sooner (at longer FID) and fought more vigorously against humans (pecked more frequently and more intensively) than females. Aggressiveness is a behavioral trait repeatedly found to be associated with boldness (Sih et al. 2004). Males usually are more aggressive than females in systems in which the male is the sex in charge of obtaining territories or where intramale competition for females is high (Wingfield et al. 1990; Schuett et al. 2010), as in the case of masked boobies (Nelson 1978). In the masked booby, agonistic encounters during territory defense are performed almost entirely by males (Kepler 1969). In other pelecaniform species, during the transition to independence, males were more aggressive than females independently of body mass (European shag, *Phalacrocorax aristotelis*; Velando 2000). Recently, it has been proposed that sexual asymmetries in behavioral strategies can be favored by sexual selection (Dingemanse and Réale 2005; Schuett et al. 2010) as, for example, male aggressiveness and boldness can be selected by females in a sexual context (Godin and Dugatkin 1996; Schuett et al. 2010). In addition, larger individuals and larger sex have superior competitive capacities for parental care and food resources (Selander 1972; Bortolotti 1986; Fargallo et al. 2003); thus, more aggressive or bolder behaviors can be favored in smaller individuals/sex to compensate their initial disadvantage against larger opponents (Drummond et al. 1991; Morrell et al. 2005).

## Coloration, behavior, and hormone relationships

Both males and females having lower plasma T levels exhibited bolder or more aggressive behaviors. Also females with smaller brown patches (whiter) showed bolder behavior. This result is contrary to the hypothesis that predicts bolder behavior in more

melanized individuals with higher levels of glucocorticoids and T (Ducrest et al. 2008). Previous studies have also found results that contradict the positive pleiotropic hypothesis. In the white-throated sparrow *Zonotrichia albicollis*, less melanized phenotypes (white-striped vs. tan-striped individuals) show higher aggressiveness, and white phenotypes have higher levels of plasma T (Tuttle 2003; Spinney et al. 2006). Whiter least auklets *Aethia pusilla* showed higher dominance status than more melanized individuals (Jones 1990). In addition, a recent study found that shyer "low-explorer" great tits showed higher concentrations of circulating T than bolder "fast-explorer" tits (van Oers et al. 2011). Van Oers et al. (2011) argue that low basal T levels could be indicative of high peak T production during stimulation (greater T elevation) in aggressive interactions (challenge hypothesis; Wingfield et al. 1990). Taking into account the lack of knowledge we have about the sensitivity level of T receptors to a given T concentration or the effect that the concentration of T receptors has in the response to basal concentrations of T, the explanations given by van Oers et al. (2011) in great tits can also be applied to masked booby fledglings. Individual differences in behaviors mediated by hormones, mainly those that cannot be explained by circulating T, may instead relate to individual differences in sensitivity to hormones in the brain, such as the abundance of androgen receptors, aromatase, or estrogen receptor alpha (Rosvall et al. 2012; Bergeon Burns et al. 2013).

Interactions among individuals affect steroids inducing T levels to be modulated by social context (Wingfield et al. 1990; Adkins-Regan 2005; Vergara and Martínez-Padilla 2012). Individuals more exposed to social interactions show elevated circulating T levels with respect to those living in more quiet or stable environments (Wingfield et al. 1990; Spinney et al. 2006; Vergara and Martínez-Padilla 2012). There is clear evidence that T secretion is associated with aggressive behavior, and although this mainly works in reproductive contexts (Koolhaas et al. 2010), T has also been inferred to play a function in the regulation of competitive behavior in young birds either within nest sibling competition or by aggressively defending territories against intruding chicks and adults (Groothuis and Meeuwissen 1992; Schwabl 1993; Ferree et al. 2004). The degree to which the involvement of the hypothalamic-pituitary-gonadal (HPG) system in aggressive behavior may be generalized to other behavioral displays and situations is unknown (Koolhaas et al. 2010). Our results reveal an association between circulating T levels and the development of behavioral strategies. We need to know whether plasma T acts as a main agent modulating responses against potential risks or whether other physiological systems implicated in animal behavior affect T secretion. HPA system profiles seem to determine the variation in circulating T and behavior (Koolhaas et al. 1999; Sapolsky et al. 2000; Korte et al. 2005). The hypothalamus produces a neuropeptide in the brain named corticotropin-releasing factor that stimulates the pituitary gland to secrete ACTH into the blood. ACTH stimulates, in turn, the adrenal cortex to release glucocorticoids (Korte et al. 2005). This adrenocortical response suppresses T secretion promoting, in general, negative correlations between T and CORT circulating concentrations (Sapolsky et al. 2000; Korte et al. 2005). In our study, no relationships were found between basal T and CORT levels or between CORT and behavior. However, to correctly evaluate the interconnection between HPA, HPG, and behavior, we need to measure the adrenocortical response to environmental perturbations, that is, how CORT concentration increases from basal to maximum levels after our manipulation and the time required to go back to initial concentration, as probably the adaptive process for actively maintaining stability through change (allostasis) can better identifies behavioral strategies.

## Sex-specific phenotypic integration

Our study indicates sex-specific differences in the individual consistency of behavioral, color, and physiological phenotypes. GLMs showed that males were bolder than females; boldness negatively correlates with melanization in females, and this trend being weaker for males. Also, both bolder males and females had lower plasma T concentration than shyer individuals. Sex differences were more evident when using SEMs. In females, boldness negatively correlated with melanization (brown patch size) and T concentration but not in males (see Figure 5), suggesting that the correlation found by GLMs between behavior and T concentration was mainly due to the effect of females. Both statistical procedures reflect sexual differences in the convergence of phenotypic traits, SEMs in addition, reveal a stronger phenotypic integration for females compared with that observed in males. This result suggests that shy–bold behavior, T concentration, and melanin-based coloration seem to have a higher functional intercorrelation in females than in males, which does not necessarily imply a higher probability of these traits to co-vary pleiotropically in females compared with males, because 1) it is unknown whether the correlation among different phenotypic traits is based on genetic correlations and 2) male traits may show a higher plasticity, which may apparently result in a lower phenotypic integration (Pigliucci and Preston 2004). In addition, a possible higher variability of measured phenotypic traits in females (particularly behavior) might facilitate to detect significant covariance in this sex. Hence, although we do not know the reason why phenotypic integration differs between sexes, our results suggest that there are sexual differences in behavioral and physiological strategies linked to color expression in a monochromatic bird species.

## Melanin-based signals in juvenile plumages

The color variation we found and its association with behavior and endocrine profiles suggest that coloration of juvenile plumage can act as an intra-age class signal of sex, body mass, and personality in a social context. The few studies investigating the adaptive function of juvenile or fledgling plumage variation have been focused in the parent–offspring context. Parents seem to respond to differences in offspring plumage coloration by adjusting their care allocation consequently (Lyon et al. 1994; Penteriani et al. 2007; Galván et al. 2008; Ligon and Hill 2010). However, juvenile plumages can also play an important role in the resource competition among young individuals during the postfledging dependence period or during juvenile life in colonial species (Jones 1990; Vergara and Fargallo 2008; Vergara et al. 2010). Benefits are postulated to be the same as in other competitive situations (Vergara and Fargallo 2008; Vergara et al. 2010), that is, by signaling dominance or individual capacities it is possible to avoid risky agonistic encounters (Rohwer 1975; Senar 2006). In the case of boobies, as in other colonial seabirds, fledglings group on the fringes of the colony returns only to the parent territories to be fed (Nelson 1978), and dominance in juveniles may prevent the food from being stolen by conspecifics when they are fed by parents (Velando 2000). Masked boobies retain their juvenile plumage during at least 2 years and start breeding mostly at the fourth year. In this species, it is common to observe permanent aggregations of young nonbreeders for feeding and resting (Nelson 1978), for which it is plausible for a social context to

play an important role in color signaling within these nonbreeder aggregations.

If the behavioral test we carried out can be interpreted within the shy–bold continuum, our study shows that color traits, apart from sex and endocrine profiles, may mirror not only aggressiveness but behavioral strategies. These results are in tune with the idea that different traits may converge in a given phenotype via pleiotropic effects of genes involved or correlated with development pathways in the expression of related characters. More specific studies are needed to disentangle possible mechanisms behind the intercorrelation of these traits, such as the phenotypic plasticity, the additional physiological systems involved, the role of social interactions on behavior and hormone profiles, or a more concise knowledge about the melanogenesis process. Our study highlights the importance of studying juvenile plumages within signaling theory, suggesting that plumages developed in early phases of life might have social implications within the juvenile age class and not only in a young–adult perspective. Finally, patterns of phenotypic integration can be viewed as varying solutions in response to natural selection (adaptations) or varying limitations in the evolution of traits (constraints), although both phenomena cannot be considered separately in the evolutionary history of organisms (Pigliucci and Preston 2004). In any case, our study identifies a functional group of characters whose integration differs between males and females, suggesting different selective pressures between sexes for the functional association among behavior, endocrine profile, and coloration.

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