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Beak colour dynamically signals changes in fasting status and parasite loads in king penguins

Quentin SCHULL[1,2*], F. Stephen DOBSON[3], Antoine STIER[1,2], Jean-Patrice ROBIN[1,2], Pierre BIZE[4] and Vincent A. VIBLANC[1,2]

[1] Université de Strasbourg, 67087 Strasbourg, France.

[2] CNRS, IPHC, UMR 7178, IPHC, 23 rue Becquerel, 67087 Strasbourg, France.

[3] Department of Biological Sciences, Auburn University, 311 Funchess Hall, Auburn, AL 36849, USA

[4] Institute of Biological and Environmental Sciences, University of Aberdeen, Scotland, UK.

*Correspondence: quentin.schull@gmail.com

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ABSTRACT

Dynamic ornamental signals that vary over minutes, hours or weeks can yield continuous information on individual condition (e.g. energy reserves or immune status), and may therefore be under strong social and/or sexual selection. In vertebrates, the colouration of integument is often viewed as a dynamic ornament, which in birds can be apparent in the beak. King penguins (*Aptenodytes patagonicus*) are monomorphic seabirds that possess conspicuous yellow-orange and ultra-violet beak spots that are used by both males and females in mate choice. We studied the dynamicity of beak spot sexual traits, and to what extent they reflected changes in individual condition in fasting king penguins and in penguins treated with an anti-parasitic drug. We also describe for the first time the maturation of this colourful ornament during the yearly catastrophic moult. On a time-scale of days to weeks, beak spot colouration changed in response to fasting and experimental changes in parasite load. Beak spot $UV_{brightness}$ decreased over a 10 days fast in breeding birds. For birds caught during courtship and held in captivity YO_{chroma} decreased after a 24 day fast. Birds that were treated with an anti-parasitic solution showed an increase in UV colouration after parasite removal. Altogether, our results show that beak spot colouration is a dynamic ornament that reflects multiple dimensions of changes in individual condition in breeding-fasting penguins.

INTRODUCTION

Darwin's theory of sexual selection has been central to evolutionary biology, providing scientists with a framework for understanding mechanisms that might lead to the evolution of individuals selecting mates that produce fitter offspring (Darwin 1871). When assessing mate

or competitor condition, animals often rely on ornamental signals that are costly to produce and/or maintain, and are therefore expected to honestly reflect individual quality (Zahavi 1975; Grafen 1990; Cotton et al. 2004; Walther and Clayton 2004). Mates may use such ornaments to assess the direct and/or indirect fitness benefits (e.g. paternal care, genetic benefits; MØller and Thornhill 1998; Mays and Hill 2004; Fromhage et al. 2009) that arise from mating with partners able to bear their cost.

In species where interactions with mates and/or social competitors occur repeatedly, there should be strong selection for dynamic signals that allow to continuous tracking of changes in individual condition over extended periods of time (Velando et al. 2006; Ardia et al. 2010; Rosenthal et al. 2012). Dynamic changes in integument colouration have been reported in fish and amphibians (Sköld et al. 2013), reptiles (Weiss 2002), mammals (Stephen et al. 2009), and birds (Velando et al. 2006; Ardia et al. 2010). In birds, studies have suggested that beak colouration may serve as a dynamic signal of individual condition (Blount et al. 2003; Faivre, Grégoire, et al. 2003; Navarro et al. 2010; Rosenthal et al. 2012). In contrast to feathers that are replaced only during moult and constitute an inert (non-vascularized) tissue, the beak is a vascularized part of the integument (Lucas and Stettenheim 1972). Thus, rapid changes in beak colouration may reflect more dynamic changes than feathers in the deposition or mobilization of pigments (e.g. carotenoids; Alonso-Alvarez et al. 2004), or rearrangement of local microstructures (e.g. keratin; Dresp and Langley 2006) linked to modifications in individual condition over time.

One important trade-off shaping the evolution of honest signals is between sexual ornaments and immune function, and by extension resistance to parasites (Hamilton and Zuk 1982). Differential allocation of pigments to ornaments or immune function is thought to act

as a constraint, so that only high quality individuals are able to invest at the same time pigments into both coloured ornaments and efficient immune defences (Blount et al. 2003; Faivre, Préault, et al. 2003; Aguilera and Amat 2007). In addition, pigment-based colouration appears to change rapidly under stressful conditions or immune stimulation (Faivre, Grégoire, et al. 2003; Rosenthal et al. 2012) raising questions about the potential role of pigmented ornaments in reflecting rapid changes in body condition and energy depletion. In addition to pigmented ornaments, structural colours such as ultra-violet (UV) may also provide information on individual quality. For instance, inter-individual variation in integument UV colouration has been linked to inter-individual variation in body condition in several species (Bize et al. 2006; Jacot and Kempenaers 2007; Dobson et al. 2008; Viblanc et al. 2016). However, because of the structural nature of UV colours, it is unclear whether this trait is labile and reflects intra-individual changes in condition over short to long time periods.

Using king penguins (*Aptenodytes patagonicus*) as a study species, we studied the dynamicity of beak colouration and tested whether it responded to ecological factors (parasites, long-term fasting) that might produce changes in beak colouration. Both male and female king penguins display colourful yellow-orange beak spots that also reflect ultraviolet (UV) (Dresp et al. 2005; Jouventin et al. 2005). In particular, beak UV appears to be an important signal of individual quality used in mutual mate choice. For instance, experimental studies have shown that reducing beak $UV_{brightness}$ decreases the pairing likelihood in both males and females (Nolan et al. 2010). Further, beak UV is associated with indices of condition. For instance, beak $UV_{brightness}$ was positively correlated to body condition in breeding males, but negatively correlated in breeding females (Dobson et al. 2008; Viblanc et

al. 2016). Beak UV_{hue} is negatively related to total oxidative damage in breeding females but not in males, and is negatively related to individuals' responsiveness to acute stress in both sexes (Viblanc et al. 2016).

UV colouration of penguin beaks is structural, resulting from the reflection of light off stacks of elongated lamellae in the horny layer of the beak, forming a photonic microstructure that reflect light in the UV to violet wavelengths (Dresp and Langley 2006). Removing 17% of the beak spot upper-layer results in a decrease of 10% in maximum reflectance. When the horn thickness is reduced by 38% the UV reflectance disappears but the YO orange reflectance remains (Dresp et al. 2005). The remaining reflectance (starting after 450 nm) being that of the yellow-orange beak colour, which is likely caused by carotenoid pigments assimilated through diet are present only in the deeper parts of the beak (McGraw et al. 2007). Indeed, yellow-orange beak colours appear to be constrained by the availability of environmental resources, birds displaying higher yellow-orange beak hue in good years (Keddar, Couchoux, et al. 2015). Because previous studies of beak spot colouration in this species have relied on measures of beak colouration taken on different individuals at a single point in time, it remains unclear whether beak colouration may signal changes in bird condition during breeding and what underlying factors might trigger short-term changes in colouration. Here, we used repeated measures on breeding adult king penguins to investigate changes in beak colouration focusing on two original aspects of variation in beak colour. First, we examined the development of beak colouration following moult. King penguins (and probably the closely related emperor penguin, which has similar UV and yellow-orange beak spots; Jouventin et al. 2005b) entirely renew the coloured keratin superficial layer on both sides of the beak at the end of process of feather moult (see

ESM1), a feature that appears unique in birds. Renewed beak spots are present by the time birds return to land after the post-moult foraging trip at sea, and are displaying for mates on the beach adjacent to the breeding colony.

Second, we studied variation in beak spot colouration in response to physiological constraints (fasting and parasite load) of key importance to king penguins during reproduction. King penguin fast on-land, facing repetitive long-term fasting periods (up to 3-5 weeks for the male during the first shift of incubation) (Groscolas and Robin 2001) and as mainly colonial animal cope with parasites which made of these two essential aspects of reproduction in king penguins. Breeding birds will abandon reproduction if their energy reserves are critically depleted (Groscolas and Robin, 2001), and strong ectoparasite loads are known to diminish the health of adults and their offspring (Gauthier-Clerc et al. 1998; Mangin et al. 2003; Bize et al., unpublish data) and as pathogen's vectors (Gauthier-Clerc et al. 1999) may be responsible of disease transmission. As bi-parental investment is an obligate condition for reproductive success, ornamental signals that dynamically reflect individual energetic reserves or parasite loads should be of importance for assessing partner condition throughout the season, and to allow birds to adjust their reproductive effort accordingly.

METHODS

Study species

We studied king penguins in the 'Baie du Marin' colony on Possession Island, Crozet Archipelago (46°25' S, 51°45' E) during the breeding season (November to March) in 2011-12, 2012-13 and 2014-15. King penguins are long-lived seabirds with a unique breeding cycle. After having moulted and replenished their energy stores at sea, males and females

will court and establish a breeding territory during a period of ca. 11 days before the female lays a single egg (Weimerskirch et al. 1992). Pairing takes place after ritualized interactions with several potential partners that include calling and exposing coloured ornaments (including the beak spot) to tentative partners in stereotyped postures (sky-pointing of the head in unison with a potential partner; Jouventin 1982). Once the egg is laid, males take duty for the first incubation shift; and must continue what is already a prolonged fasting period. The female relieves them some 16 days later, and males then return at sea to forage (Weimerskirch et al. 1992). Alternated incubation continues until the egg hatches on average 54 days later (Stonehouse 1960). Birds followed in these studies were at least 3-4 years old, the age at first reproduction in king penguins (Stonehouse 1960), but their exact age in unknown.

Measures of beak spot colouration

Colours reflected by the beak spot were measured using a portable JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) with a spectral resolution of 0.3 nm across the spectral range of 320-700 nm. The JAZ contains a pulsed-xenon light and was calibrated against a white standard (Ocean Optics Spectralon). Measures were repeated 3 times across each beak spot (in the yellow-orange region) using a 200 µm fibre-optic probe with a 90° angle window. The obtained spectra were smoothed and averaged using an R script adapted from Montgomerie (Montgomerie 2008) before calculating mean brightness, hue, and chroma (see below) over the spectral range 320-700 nm, which corresponds to the full range of spectral sensitivity in birds (Cuthill 2006). The reflectance spectrum of king penguin beak spots is composed of a peak in UV-violet region and a plateau in the yellow-orange region of

the spectrum (Fig. 2). For the UV part of the spectrum, the average wavelength of maximum reflectance (i.e. the peak maxima) is around 390 nm, but may reach as far as 420 nm when hydrated (Dresp and Langley 2006). Whereas, Keddar and colleagues split the entire spectrum into two parts (before and after 499 nm) in order to separate UV and yellow-orange domains (Keddar et al. 2013; Keddar, Jouventin, et al. 2015), the UV peak of birds in our study extended largely over 450 nm (see Fig. 2), ending at around 490 nm (which also corresponded to the start of the yellow-orange beak colour). Thus, to avoid missing a part of light/information emitted by the microstructure for the UV signal and to avoid integrating light in the YO domain that actually comes from a structural rather than pigment based colouration, we calculated colour variables separately over those 2 regions: 320-490 nm for UV and 491-700 nm yellow-orange (YO) colours. The spectral intensity of the beak spot, mean UV-violet brightness ($UV_{brightness}$) and mean yellow-orange brightness ($YO_{brightness}$) were calculated by averaging reflectance over wavelengths 320-490 nm and 491-700 nm, respectively (Montgomerie 2006). Hue is a measure of colour appearance (e.g. 'blue', 'yellow', etc.). For the yellow-orange plateau portion of the spectrum, YO_{hue} was calculated as the wavelength at which the reflectance was halfway between its maximum and minimum (Keddar et al. 2013). For the UV-violet peak, UV_{hue} was calculated as the wavelength of maximum reflectance between 320 and 490 nm. Finally, chroma is a measure of colour purity and was calculated within the region of interest (UV_{chroma} and YO_{chroma}) as the difference between maximum and minimum reflectance over the mean reflectance for that particular region (formula S_8; Hill and McGraw 2006, p. 108). Repeatability of the colour measurements are high (between 0.70 and 0.91) and given in ESM 1. Correlations between those spectral parameters are presented in Fig. 1a (see also Viblanc et al. 2016). Briefly, the

correlations between UV and YO colour parameters are very low, consistent with the fact that those colours in the king penguin are produced by two independent mechanisms (structural for the UV and pigmentary for YO colours; see Dresp et al. 2005). For UV colours, brightness, hue and chroma parameters were independent and therefore we considered them separately in further analyses. For YO colours however, those parameters were highly correlated. Thus, we chose to focus YO_{chroma} for the following reasons. First, it has been argued that the signal with the highest among-individual variance also contains the most information (Dale 2006). YO_{chroma} was the parameter the most correlated with both brightness and hue, and also that presenting the highest Coefficient of Variation (Fig. 1b). Second, YO_{chroma} has been shown to directly reflect ornament pigment concentration in several species (Saks et al. 2003; McGraw and Gregory 2004).

Changes in beak spot colouration following the moult

For adult king penguins, beak spots are renewed each year at the end of the period of moult of the entire plumage (ESM 2). Moulting of feathers and beak spots occur before the start of the breeding season in November-January and the whole process takes ca. 32 days (Groscolas and Cherel 1992). To study the maturation of the new beak spot following the moult process, 25 moulting males were caught shortly before the end of moult and kept captive in a pen. Birds were checked daily for moult completion, and the colouration of their beak spot was measured before the moult started and after the moult was completed. Seventeen birds were kept captive for an extra 2 days, (5 were released because were close to reaching a critical body mass, i.e. phase 3 of fasting; Groscolas and Robin 2001) and the colouration of the beak spot was measured a second time. All birds were released and left at

sea to forage. Before being released, birds were identified by marking them on the belly with a unique letter/number combination using a non-toxic human hair dye (Franck Provost, blue-black 2.1). The beach was walked every day to search for birds that returned to the colony to start breeding after having being at sea to feed and replenish their body reserves. Eighteen of the 25 followed birds were caught on the beach within a few hours after returning from their foraging trip and beak measurements were taken a final time. At the end of the moult, 3 individuals presented no reflectance signal whatsoever in the UV part of the beak (completely flat spectrum) but a classical YO reflectance spectrum, clearly due to a lack of maturation of the UV component. These 3 individuals were not taken into account in the analyses of UV colour parameters, explaining the variation of the sample size between UV and YO colour analyses (i.e. $N = 22$ vs. 25).

We studied changes in beak colouration following the moult by specifying beak colour variables (hue, chroma and brightness) as dependent variables in separate Linear Mixed Models (LMMs). The time period of colour measurement was entered as a discrete ordinal variable with 3 levels: the day the old beak spots were shed (day 0), two days later (day 2), and the day birds returned from their post-moult foraging trip at sea (courtship). Bird ID was entered as a random effect to account for repeated measurements on individual birds. Correlation between colour parameters before moult and after their post-moult foraging trip (courtship) were investigate using Spearman's rank test on 16 birds for which we had both measurements.

Changes in beak colouration during fasting

We investigated the effect of fasting on beak spot colouration using either breeding penguins that were naturally fasting while incubating their egg or of captive penguins that were forced to fast. We ran three different studies that covered different fasting and duration periods for this species.

In a first study performed in 2011-12, we studied 22 males and 22 females during the third and fourth incubation shift, respectively. We measured beak spots directly in the colony while birds were incubating their eggs. Birds were measured on their second day of incubation and again 6 days later (incubation day 8). We waited 2 days before the first measure to insure that individuals had settled on their eggs, thus avoiding any risk of breeding abandonment. Those birds were all returning from their foraging trip before taking their incubation shift, and thus this study covers changes in beak colouration during the first days of fasting.

In a second study performed in 2014-15, we investigated changes in beak colouration of 36 breeding males during their first incubation shift. Beak colouration was measured on the third day after the start of incubation and again 10 days later, i.e. on day 13 of incubation. Males do not return at sea to feed between the period of courtship display and their first incubation shift, and thus they had already endured at least 10-days of fasting before our first measurement of colouration. Hence, this study covers changes in beak colouration during the second half of a long (> 20 days) fasting period.

Finally, in a third study performed in 2013-14 and 2014-15, we caught 20 males during courtship on land (10 birds each year) and kept them captive in a pen. These individuals experienced a forced fasting period (0-24 days) covering the natural fasting periods of our first (2-8 days) and second (ca. 13-23 days) studies presented above. Such

prolonged fasting periods are well within the natural range of fasting observed in this species. In these birds, we measured body mass and beak spot colouration every 6 days to investigate changes in colour as fasting progressed. Upon release, all birds were observed departing to sea to feed and subsequently seen returning at the colony to breed.

Within each experiment, monitored birds were all caught at same day of the same breeding shift and thus had comparable breeding status. Changes in beak colouration during fasting were investigated by specifying beak colour variables (hue, chroma and brightness) as dependent variables in separate LMMs. The time period of colour measurement was entered as a discrete ordinal variable (e.g. day 2 and day 8 in the first study). Bird sex was entered as a fixed factor in the models, and for the first experiment (the only one where both sexes were monitored), we considered the interaction of sex and time. However, as the interaction was not significant, we removed it from the final model. In addition, we accounted for body girth, a proxy to body condition (Viblanc et al. 2012), as a covariate in the models. Again however, as body girth never significantly affected colour parameters, we removed it from the final models. Bird ID was entered as a random effect to account for repeated measurements on individual birds. In the third study, captive birds were measured in two different years (2013-2014 and 2014-2015), and thus we entered the year as random factor to control for potential year effects on colouration (Keddar, Couchoux, et al. 2015).

Response of beak colouration to experimental parasite removal

In 2011-2012, we investigated the effects of seabird parasite loads on beak colouration by experimentally removing parasites in an experimental group of 20 breeding pairs using the anti-parasitic solution Eprinex Pour-On®. This solution is commonly used in cattle and

poultry and known to remove a large spectrum of ecto- and endo-parasites including worms, lice, ticks, mange mites and grubs) (Shoop et al. 1996). A control group of 20 pairs was treated with a solution of propylene glycol, which is the solvent used in Eprinex Pour-On®. We obtained information on changes in beak spot colouration in 27 treated birds (14 males, 13 females) and 33 control birds (16 males, 17 females). To control for possible confounding effects of breeding timing and localization in the colony on inter-individual variation in beak spot colouration, we applied our treatments so that treated and control pairs did not differ in their breeding onset (all were early breeders) or where they were breeding in the colony. The anti-parasitic and control solutions were deposited on the skin of the birds just below the feathers at the base of the neck at the beginning of the first incubation shift and of the third incubation shift in males and at the beginning of the second and fourth incubation shift in females. The efficiency of the anti-parasitic treatment was controlled by counting tick loads (*Ixodes uriae*) on a small part of the body (the head) of monitored birds. At the start of the treatments (i.e. beginning of shift one of males and shift two of females), there was no difference in the number of ticks on the head of birds treated with the anti-parasitic versus sham solutions (mean ± s.e. = 2.26 ticks ± 0.62 versus 1.39 ticks ± 0.59; Wilcoxon's test: $chi^2 = 0.21$, $df = 1$, $P = 0.64$). The Eprinex Pour-On® solution was efficient at removing parasites as reflected by lower tick loads after treatment on the head of birds treated with the anti-parasitic versus sham solutions (0.05 ticks ± 0.64 versus 3.15 ticks ± 0.62; *Wilcoxon's test*: $chi^2 = 27.23$, $df = 1$, $P < 0.001$). Effects of our treatments on changes in beak spot colouration were measured during shift 3 and 4 of males and females, respectively, by taking a first measure of beak colouration on the second day after the start of incubation and again 6 days later, i.e. on day 8 of incubation.

Changes in beak colouration in response to our treatments were investigated by specifying beak colour variables (hue, chroma and brightness) as dependent variables in separate LMMs. The effect of the experimental anti-parasitic treatment on beak colouration was tested from the significance of the interaction between treatment (anti-parasitic vs. sham) and time period (day 3 vs. day 8) in the model. Sex was added as a fixed factor in the model to test for potential sex differences in beak colouration variation and its interaction with treatment was also considered. Again, body girth was added as a covariate in the model and its interaction with treatment and period considered, but as it never showed any significant effect on colour parameter, it was removed from the final models. Bird ID was entered as a random effect to account for repeated measurements on individual birds.

Statistics

Statistical analyses were run with R v.3.1.1 (R development core team). F-statistics for fixed effects (tests of differences from zero), the total number of observations (n) and corresponding number of individuals (N) are given. Effects were considered significant for $P < 0.05$. When appropriate, significant differences between groups were assessed using Tukey's Honest Significant Difference (HSD) tests for least square means. We insured residuals followed a normal distribution using qqplots (opposing theoretical Quantiles to Sample Quantiles).

Ethical statement

All experiments were approved by an independent ethics committee (Comité d'éthique Midi-Pyrénées pour l'expérimentation animale) commissioned by the French Polar

Institute and comply with the current laws of France. Authorizations to enter the breeding colony and handle the birds were provided by the "Terres Australes et Antarctiques Françaises" (permit n°2010-65 issued on the 3rd of September 2010, n°2011-96 issued on the 14th of October 2011, n°2012-116 issued on the 29th of October 2012, n°2013-72 issued on the 29th of October 2013 and n°2014-127 issued on the 15th of October 2014).

RESULTS

Changes in beak spot colouration following the moult

Birds showed pronounced changes in the colouration of their beak spot following the moult. The new beak spot appeared particularly pinkish and changed from day to day (Fig. 2). $UV_{brightness}$ of the new beak decreased by 14% within the first 2 days of shedding the old beak spot, and continued to decrease by another 23% during the post-moult foraging trip at sea (LMM; F =79.21, df = 2, P < 0.001, n = 55, N = 22). Freshly moulted birds exhibited a rapid decrease in the UV_{hue} of their new beak spot (by 20.7 nm on average) within the first 2 days of shedding the old beak spot (Fig. 2). UV_{hue} continued to decrease during the post-moult foraging trip (another 10.7 nm on average), albeit less substantially (F = 30.20, df = 2, P < 0.001, n = 55, N = 22). We observed increases both in UV_{chroma} (+19% within 2 days post-moult and another 23% by the time the birds returned for courtship; F = 51.04, df = 2, P < 0.001, n = 55, N = 22) and YO_{chroma} (+24% within 2 days post-moult, and another 13% by courtship; F = 23.88, df = 2, P < 0.001, n = 60, N = 25).

When compared colour parameters before moult and after birds came back from their post-moult foraging trip, UV_{hue} was significantly correlated (Spearman's rank correlation; *rho*

= 0.64, S = 246.7, P = 0.008, n = 30, N = 15, Fig. 3), other parameters remained uncorrelated (-0.36 < *rho* < 0.38, 422 < S < 992, 0.148 < P < 0.51, n = 30, N = *15*).

Changes in beak spot colouration during fasting

In breeding penguins naturally fasting in the colony, we found no significant changes in beak spot colouration during the first days of fasting (between day 2 and 8 of males in shift three and females in shift four) (LMMs; 0.01 < F < 0.51, 0.48 < P < 0.98, n = 88, N = 44). In contrast, we found a significant decrease of 6 % in $UV_{brightness}$ in breeding male penguins at the end of a long natural fasting period (between day 13 and 23 of the first incubation shift) (LMM; F = 7.90, P < 0.01, n = 72, N = 36, Fig. 4) and no significant changes for the other colour parameters (LMMs; 0.16 < F < 2.54, 0.12 < P < 0.69, n = 72, N = 36).

In captive birds that fasted up to a similar body mass of that naturally occurring at partner relief (24 days), fasting duration did not affect UV beak colouration (brightness, hue or chroma) (LMMs: 0.92 < F < 2.04, df = 4, 0.10 < P < 0.46, n = 91, N = 20). In contrast, YO_{chroma} decreased (F = 4.23, df = 4, P < 0.05) with increased fasting (Fig. 5), and showed a strong significant decrease at the end of the fasting period (Tukey's HSD: -3.63 < Z < -2.77, 0.003 < P < 0.044, ESM 3).

Response of beak colouration to experimental parasite removal

Six days after the experimental treatments (Eprinex® or sham), birds treated with the anti-parasitic solution showed significant increases in beak $UV_{brightness}$ and UV_{hue}, and a decrease in UV_{chroma} compared to before the treatments (Table 1; Fig. 6). Control birds did not show significant changes in beak colouration after having received the placebo (Tukey's HSD;

0.80 < P < 0.99, n =120, N = 60). Bird sex, whether considered independently or in interaction with treatments and/or time period, never significantly affected beak colouration (0.12 < P < 0.96 n = 120, N = 60). The effect of sex was removed from the final models presented in Table 1.

DISCUSSION

Dynamic ornamental signals can provide continuous information on individual condition, and are expected to be under strong sexual and social selection (e.g. Velando et al. 2006). In birds, beak colouration has been proposed to function as a dynamic ornament (Faivre, Grégoire, et al. 2003; Navarro et al. 2010; Rosenthal et al. 2012)as indeed appears to be the case in our species. King penguins showed a rapid maturation of beak colouration following the moult and we found dynamic changes in colouration in response to changes in individual condition (fasting status and parasite load) in breeding birds over the scale of a single breeding season.

Changes in beak spot colouration following the moult

Although studies on avian moult are numerous, there is virtually no information on moulted structures other than feathers (King and Murphy 1990). Horn, claw and beak material are thought to grow continuously in response to wear, but king penguins appear to be an exception in that the entire horny material of their beak spot is shed every year at the end of the moult, while the rest of the black beak horn is not. Thus, beak spots are replaced each time these long-lived seabirds breed, most of the time attracting a new breeding partner (Bried et al. 1999; Olsson 1998). After the moult, beak spots colouration becomes less bright

changing to a deeper (decrease in hue) and purer UV colour (Fig. 2, ESM4). As previously mentioned, the UV colouration of penguin beaks is structural, resulting from the reflection of stacks of elongated lamellae (multiple layers of doubly folded membranes) in the horny layer of the beak (Dresp and Langley 2006). The distance (in nm) separating those double-folds is responsible for UV_{hue}, i.e. the lattice dimension of the photonic crystals (Dresp and Langley 2006). This photonic property may be explained by Bragg's law, with $\lambda\ max = n2d\ sin\theta$, where $\lambda\ max$ is the peak wavelength of reflected light, n is the average refractive index of the tissue, d is the separation of the layers (lattice dimension), and θ is the angle of incidence of the light (Bragg 1915). Thus, our results suggest that as the beak matures, there is a decrease in the distance between the doubly folded membrane structures that compose the upper-layer of the beak. Simultaneously, although the magnitude of change is weaker, the yellow-orange colour of the beak also becomes purer as chroma increases. These latter colour changes occur as the bird prepares to mate, over a period of 13-20 days and are likely explained by the deposition of carotenoid pigments in the deeper layers of beak spots (higher chroma), as is the case in many bird species (Saks et al. 2003; McGraw and Gregory 2004). Interestingly, comparing colours before and at courtship for the same birds, UV_{hue} appeared strongly correlated (Fig. 3). It suggest that UV_{hue} (distance between doubly-folded membranes) is determined largely genetically, but some scope for variation does exist (i.e. time since the last moult, time spent at sea during the past year, time spend preening, etc.

Beak spot colouration and fasting

Nutritional status and body condition are important information that may be used by conspecifics both in reproductive and social contexts. In the Alpine swift and the European

starling for instance, parents adapt their feeding effort to the UV colouration of chick skin, which is positively correlated with body mass and structural size, and is used by parents to adapt their feeding behaviour to the intensity of the reflected colour of their chicks (Bize et al. 2006). In the same way, in blue-footed boobies, rapid experimental changes in male foot integument colouration (reflecting nutritional status) elicited rapid adjustments in female reproductive strategies, i.e. facilitation of brood reduction by laying smaller eggs compared to controls (Velando et al. 2006). In our study, male beak colouration appeared to reflect changes in nutritional status. Over 24 fasting days, captive males showed a progressive decline in YO_{chroma} but no change in UV colouration. However, when fasting was prolonged for even longer periods in colonial conditions (over >24 days including 11-15 days of courtship followed by 13 days of incubation; Fig 4), $UV_{brightness}$ appeared to decrease. Those results suggests that UV and YO colouration may signal fasting status on different time-scales. A progressive decrease in YO_{chroma} in fasting birds may reflect a re-allocation of beak pigments to antioxidant defences, in line with the hypothesis of costly signalization by (limited) carotenoid-dependent structures (e.g. in birds: Alonso-Alvarez et al. 2004; in fish: Pike et al. 2010; but see Cote et al. 2010 in reptiles). This suggestion is consistent with our recent data suggesting that plasmatic anti-oxidant defences indeed increase over the course of fasting (QS, FC, VAV, AS, JPR and PB; *unpublished data*). Yellow-orange colours are often produced by exogenous carotenoid pigments, which are acquired from the diet and may reflect yearly environmental forage conditions (Linville and Breitwisch 1997; McGraw et al. 2009; Slagsvold and Lifjeld 2009). In king penguins, similar mechanisms might explain variation in YO colour production between years (i.e. higher YO colour in years of high resource availability; Keddar, Couchoux, et al. 2015). Decreases in $UV_{brightness}$ only at

advanced fasting stages may suggest a cost for UV maintenance and the inability to maintain high UV reflectance when energy is critically limiting. For instance, preening and associated comfort behaviour (keeping the beak clean) is generally costly in birds (Walther and Clayton 2004), including king penguins (Viblanc et al. 2011), and reducing those behaviour may allow substantial savings at advanced stages of fasting. Whether such behavioural changes linked to fasting status might indirectly affect the maintenance of beak colouration remains to be tested. Furthermore, as captive birds and free-living breeders also differed in their social environmental and breeding status, we cannot exclude that such differences also affected changes in beak colouration differently between the two groups. Further investigations are needed to clarify how beak spot dynamics are conditioned by the rate of nutrient reserves mobilization and availability of dietary antioxidants such as carotenoids by following plasmatic availability concomitantly to changes in colouration.

Beak spot colouration and parasites

Parasites drain resources (including pigments and nutrients) from their hosts, which might otherwise be partly allocated to producing ornaments. Furthermore, in fighting parasites, hosts also mount immune responses that divert resources from other functions such as ornamentation (Rosenthal et al. 2012; Velando et al. 2014). Regardless of the underlying mechanism, parasitism should affect ornamental signals (e.g., parasite-mediated sexual selection; Hamilton and Zuk 1982). Consistent with this hypothesis, we found that removing parasites from males and females during incubation produced significant changes in the UV component of beak colouration. While not influencing YO colours, removing parasites resulted in beak spots of higher $UV_{brightness}$, higher UV_{hue} and lower $UV_{saturation}$ (Fig. 6). Birds

relieved of parasites should have a less stimulated immune system, and may therefore invest more into beak colouration. Accordingly, several studies in birds have highlighted strong links between UV ornamentation and parasite load (Hõrak et al. 2001; Mougeot et al. 2010) or in a broader context immune-competence (Griggio et al. 2010; Peters et al. 2008).

Here again, the independent changes we observe in UV or YO colouration support previous findings that in king penguins, UV and YO colours of the beak are produced by distinctly mechanisms (Dresp and Langley 2006), and appear to change over the course of the breeding season. Indeed, YO carotenoid-based colouration is produced by pigments embedded in the deeper beak layers and was relatively unaffected in our anti-parasite treatment. In contrast, changes in beak UV suggest structural modifications. Since UV reflectance depends on the thickness of the upper beak layer composed of the doubly folded membrane structures, which result from the differentiation of basal cells into dead keratin (as is the case for skin renewal) (Dresp and Langley 2006), an increase in UV reflectance following parasite removal suggests either an increase in cell division or a reorganisation of those structures. However, whether the thickness of the upper beak layer can be increased after the moult (i.e. complete renewal of the structure), or whether it can be remodelled is currently unknown and requires further investigations. Moreover, ectoparasites are known as vectors of disease such as ticks with Lyme Disease (Gauthier-Clerc et al. 1999), which would not be cured with our anti-parasitic treatment and could limit the effect of our experimental treatment. Whereas other proximal mechanisms are likely, our results point to parasitism as an important influence on structural based UV colour signalisation in the king penguin.

Concluding remarks

Beak colouration of king penguins can be highly flexible, with both components of beak colouration (UV and YO) modified independently. Both hue and chroma of the UV, and chroma of the YO of beak spot appear to have a maturation process, with an associated decline in beak spot brightness that continues through pre-breeding moult, subsequent feeding at sea, return to the breeding grounds, and mating. Importantly, due to its dynamicity, beak colouration may serve as an important signal of short-term changes in individual condition over the course of a single breeding season. This supports the idea that the information conveyed by sexual ornaments is not restricted to the single time period when mate choice occurs (e.g. Velando et al. 2006; Ardia et al. 2010).

Previous studies have found that higher beak $UV_{brightness}$ is associated with higher mating prospects (Nolan et al. 2010) and different body condition in males and females (Dobson et al. 2008; Viblanc et al. 2016), and higher beak UV_{hue} is negatively related with hormonal stress responses and oxidative damages in female (Viblanc et al. 2016). In line with these studies, our current results highlighted positive associations between $UV_{brightness}$ and YO_{chroma} and fasting, when condition decrease colour decrease. In the contrary, when during breeding parasite are removed the condition of the birds increase as well as $UV_{brightness}$. The next step is to focus on how such variations might be perceived by the mate at the time it takes over egg or chick-guarding duties, how beak colouration may help parents coordinate their efforts throughout the breeding season, and how dynamic changes in beak colouration may be perceived by social conspecifics.

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TABLES

Table 1. Mixed model estimates (± SE) for the effects of an anti-parasitic treatment on beak colouration in breeding king penguins (*Aptenodytes patagonicus*). The control [C] level for the treatment factor is tested against the treatment level [T]. The time period [post-treatment] is tested against the level [pre-treatment]. Bird ID were entered as random factors in the model to account for repeated measures on the individual. Values are significant for $P < 0.05$. Note the significant interaction terms revealing different treatment effects on pre- and post-treatment measures of treated vs. control birds. n = 120 observations, N = 60 birds.

FIGURE CAPTIONS

Fig 1. a) Pairwise correlation plots between the different colour parameters in king penguin beak spots. Spearman correlation coefficients are provided and the distribution of the each colour parameter is presented. Note that UV and YO colour parameters are weakly correlated b) Coefficient of variation of the different YO colour parameters. We compiled first measurements of the 181 individuals (36 where in courtship and 145 at the beginning of their breeding shift).

Fig. 2. Changes in the beak colouration of adult king penguins (*Aptenodytes*

***patagonicus*) following the moult.** Average changes in raw spectral data over all birds are presented for illustrative purposes. Beak colour was measured on the day the old beak spot was shed (day 0), 2 days later (day 2), and after birds returned from their post-moult foraging trip for breeding (courtship). Changes in brightness, hue and chroma were assessed using LMMs, with bird ID specified as a random variable. Least-Square means ± SE are presented. Values not sharing a common letter are significantly different for $P < 0.05$. The sample sizes are given below the means.

Fig. 3 Correlation between UV_{hue} before the moult and after the post-moult foraging trip at the time the birds come for courtship. Correlation were assessed using Spearman's rank correlation test (*rho* = 0.64; $S = 246.7$; $P = 0.008$).

Fig. 4 Changes in beak $UV_{brightness}$ and YO_{chroma} for 36 male king penguins (*Aptenodytes patagonicus*) that were measured on day 3 of their first incubation shift and on day 13 after having experienced a 10-day fast while breeding in the colony. Changes in brightness were assessed using LMMs, with bird ID specified as a random variable. Least-Square (LS) means ± SE are presented. Values not sharing a common letter are significantly different for $P < 0.05$. The sample sizes are given below the means.

Fig. 5 Changes in yellow-orange beak chroma (YO_{chroma}) in 20 males that endured a prolonged fast in captivity. Changes in chroma were assessed using LMMs, with bird ID and year specified as a random variable.

Fig. 6. Interactive effects of an experimental anti-parasitic treatment on the beak colouration of adult kings penguins (*Aptenodytes patagonicus*) freely incubating in the colony. Changes in brightness, hue and chroma were assessed using LMMs, with bird ID specified as a random variable, the treatment (treated vs. sham), the time period (pre-treatment vs. post-treatment) and the interaction between those factors specified as independent variables. Least-Square (LS) means are presented. Values not sharing a common letter are significantly different for $P < 0.05$.

Table 1

	Term	Estimate ± SE	t Ratio	Prob > |t|
$UV_{brightness}$	Intercept	18.56 ± 0.57	32.99	<0.001*
	Period[Post-treatment]	-0.52 ± 0.66	-0.78	0.437
	Treatment [T]	-0.23 ± 0.84	-0.27	0.787
	Period*Treatment	4. 68 ± 0.98	4.79	<0.001*
UV_{hue}	Intercept	388.65 ± 1.51	256.57	<0.001*
	Period[Post-treatment]	0.12 ± 1.10	0.11	0.915
	Treatment [T]	-1.34 ± 2.26	-0.61	0.54
	Period*Treatment	4.15 ± 1.62	2.56	<0.001*
UV_{chroma}	Intercept	1.33 ± 0.03	46.21	<0.001*
	Period[Post-treatment]	-0.02 ± 0.04	-0.43	0.666
	Treatment [T]	0.08 ± 0.04	1.87	0.064
	Period*Treatment	-0.12 ± 0.05	-2.20	0.032*
YO_{chroma}	Intercept	1.16 ± 0.04	28.27	<0.001*
	Period[Post-treatment]	-0.01 ±0.06	-0.13	0.898
	Treatment [T]	0.13 ±0.06	2.16	0.032*
	Period*Treatment	-0.10 ±0.08	-1.249	0.217

LAY SUMMARY

Elaborate animal ornaments may inform conspecifics of changes in individual condition, particularly important in social/sexual contexts. In mutually ornamented male and female king penguins, we highlight rapid changes in beak coloration in response to individual condition (nutritional status and parasite loads). Those highly dynamic changes especially concerned structural aspects of coloration (UV reflectance of keratin microstructures) particularly important for mutual mate choice in penguins.