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**Beak colour dynamically signals changes in fasting status  
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3 **1 Beak colour dynamically signals changes in fasting status and parasite loads in king**  
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5 **2 penguins**  
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33  
34 14 Keywords: dynamic ornament, parasites, fasting, honest signal, king penguin, sexual  
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## 16 ABSTRACT

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

## 35 INTRODUCTION

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

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3 39 or competitor condition, animals often rely on ornamental signals that are costly to produce  
4  
5 40 and/or maintain, and are therefore expected to honestly reflect individual quality (Zahavi  
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8 41 1975; Grafen 1990; Cotton et al. 2004; Walther and Clayton 2004). Mates may use such  
9  
10 42 ornaments to assess the direct and/or indirect fitness benefits (e.g. paternal care, genetic  
11  
12 43 benefits; MØller and Thornhill 1998; Mays and Hill 2004; Fromhage et al. 2009) that arise  
14  
15 44 from mating with partners able to bear their cost.

17 45 In species where interactions with mates and/or social competitors occur repeatedly,  
18  
19 46 there should be strong selection for dynamic signals that allow to continuous tracking of  
20  
21 47 changes in individual condition over extended periods of time (Velando et al. 2006; Ardia et  
22  
23 48 al. 2010; Rosenthal et al. 2012). Dynamic changes in integument colouration have been  
24  
25 49 reported in fish and amphibians (Sköld et al. 2013), reptiles (Weiss 2002), mammals  
26  
27 50 (Stephen et al. 2009), and birds (Velando et al. 2006; Ardia et al. 2010). In birds, studies have  
28  
29 51 suggested that beak colouration may serve as a dynamic signal of individual condition  
30  
31 52 (Blount et al. 2003; Faivre, Grégoire, et al. 2003; Navarro et al. 2010; Rosenthal et al. 2012).  
32  
33 53 In contrast to feathers that are replaced only during moult and constitute an inert (non-  
34  
35 54 vascularized) tissue, the beak is a vascularized part of the integument (Lucas and Stettenheim  
36  
37 55 1972). Thus, rapid changes in beak colouration may reflect more dynamic changes than  
38  
39 56 feathers in the deposition or mobilization of pigments (e.g. carotenoids; Alonso-Alvarez et al.  
40  
41 57 2004), or rearrangement of local microstructures (e.g. keratin; Dresp and Langley 2006)  
42  
43 58 linked to modifications in individual condition over time.

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46 59 One important trade-off shaping the evolution of honest signals is between sexual  
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48 60 ornaments and immune function, and by extension resistance to parasites (Hamilton and Zuk  
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50 61 1982). Differential allocation of pigments to ornaments or immune function is thought to act  
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4 62 as a constraint, so that only high quality individuals are able to invest at the same time  
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6 63 pigments into both coloured ornaments and efficient immune defences (Blount et al. 2003;  
7  
8 64 Faivre, Pr  ault, et al. 2003; Aguilera and Amat 2007). In addition, pigment-based colouration  
9  
10 65 appears to change rapidly under stressful conditions or immune stimulation (Faivre,  
11  
12 66 Gr  goire, et al. 2003; Rosenthal et al. 2012) raising questions about the potential role of  
13  
14 67 pigmented ornaments in reflecting rapid changes in body condition and energy depletion. In  
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16  
17 68 addition to pigmented ornaments, structural colours such as ultra-violet (UV) may also  
18  
19 69 provide information on individual quality. For instance, inter-individual variation in  
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21 70 integument UV colouration has been linked to inter-individual variation in body condition in  
22  
23 71 several species (Bize et al. 2006; Jacot and Kempenaers 2007; Dobson et al. 2008; Viblanc et  
24  
25 72 al. 2016). However, because of the structural nature of UV colours, it is unclear whether this  
26  
27 73 trait is labile and reflects intra-individual changes in condition over short to long time  
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29 74 periods.  
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34 75 Using king penguins (*Aptenodytes patagonicus*) as a study species, we studied the  
35  
36 76 dynamicity of beak colouration and tested whether it responded to ecological factors  
37  
38 77 (parasites, long-term fasting) that might produce changes in beak colouration. Both male and  
39  
40 78 female king penguins display colourful yellow-orange beak spots that also reflect ultraviolet  
41  
42 79 (UV) (Dresp et al. 2005; Jouventin et al. 2005). In particular, beak UV appears to be an  
43  
44 80 important signal of individual quality used in mutual mate choice. For instance, experimental  
45  
46 81 studies have shown that reducing beak  $UV_{\text{brightness}}$  decreases the pairing likelihood in both  
47  
48 82 males and females (Nolan et al. 2010). Further, beak UV is associated with indices of  
49  
50 83 condition. For instance, beak  $UV_{\text{brightness}}$  was positively correlated to body condition in  
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52 84 breeding males, but negatively correlated in breeding females (Dobson et al. 2008; Viblanc et  
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85 al. 2016). Beak  $UV_{hue}$  is negatively related to total oxidative damage in breeding females but  
86 not in males, and is negatively related to individuals' responsiveness to acute stress in both  
87 sexes (Viblanç et al. 2016).

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

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3 108 ESM1), a feature that appears unique in birds. Renewed beak spots are present by the time  
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6 109 birds return to land after the post-moult foraging trip at sea, and are displaying for mates on  
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8 110 the beach adjacent to the breeding colony.  
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11 111 Second, we studied variation in beak spot colouration in response to physiological  
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13 112 constraints (fasting and parasite load) of key importance to king penguins during  
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15 113 reproduction. King penguin fast on-land, facing repetitive long-term fasting periods (up to 3-  
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17 114 5 weeks for the male during the first shift of incubation) (Groscolas and Robin 2001) and as  
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20 115 mainly colonial animal cope with parasites which made of these two essential aspects of  
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22 116 reproduction in king penguins. Breeding birds will abandon reproduction if their energy  
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24 117 reserves are critically depleted (Groscolas and Robin, 2001), and strong ectoparasite loads  
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27 118 are known to diminish the health of adults and their offspring (Gauthier-Clerc et al. 1998;  
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29 119 Mangin et al. 2003; Bize et al., unpublished data) and as pathogen's vectors (Gauthier-Clerc et  
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31 120 al. 1999) may be responsible of disease transmission. As bi-parental investment is an obligate  
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34 121 condition for reproductive success, ornamental signals that dynamically reflect individual  
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36 122 energetic reserves or parasite loads should be of importance for assessing partner condition  
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39 123 throughout the season, and to allow birds to adjust their reproductive effort accordingly.  
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## 42 43 125 **METHODS**

### 44 45 126 **Study species**

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48 127 We studied king penguins in the 'Baie du Marin' colony on Possession Island, Crozet  
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50 128 Archipelago (46°25' S, 51°45' E) during the breeding season (November to March) in 2011-  
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52 129 12, 2012-13 and 2014-15. King penguins are long-lived seabirds with a unique breeding  
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55 130 cycle. After having moulted and replenished their energy stores at sea, males and females  
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3 131 will court and establish a breeding territory during a period of ca. 11 days before the female  
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5 132 lays a single egg (Weimerskirch et al. 1992). Pairing takes place after ritualized interactions  
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8 133 with several potential partners that include calling and exposing coloured ornaments  
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10 134 (including the beak spot) to tentative partners in stereotyped postures (sky-pointing of the  
11  
12 135 head in unison with a potential partner; Jouventin 1982). Once the egg is laid, males take  
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15 136 duty for the first incubation shift; and must continue what is already a prolonged fasting  
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17 137 period. The female relieves them some 16 days later, and males then return at sea to forage  
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20 138 (Weimerskirch et al. 1992). Alternated incubation continues until the egg hatches on average  
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22 139 54 days later (Stonehouse 1960). Birds followed in these studies were at least 3-4 years old,  
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24 140 the age at first reproduction in king penguins (Stonehouse 1960), but their exact age in  
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26 141 unknown.  
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### 143 **Measures of beak spot colouration**

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

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3 154 the spectrum (Fig. 2). For the UV part of the spectrum, the average wavelength of maximum  
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5 155 reflectance (i.e. the peak maxima) is around 390 nm, but may reach as far as 420 nm when  
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8 156 hydrated (Dresp and Langley 2006). Whereas, Keddar and colleagues split the entire  
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10 157 spectrum into two parts (before and after 499 nm) in order to separate UV and yellow-orange  
11  
12 158 domains (Keddar et al. 2013; Keddar, Jouventin, et al. 2015), the UV peak of birds in our  
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14 159 study extended largely over 450 nm (see Fig. 2), ending at around 490 nm (which also  
15  
16 160 corresponded to the start of the yellow-orange beak colour). Thus, to avoid missing a part of  
17  
18 161 light/information emitted by the microstructure for the UV signal and to avoid integrating  
19  
20 162 light in the YO domain that actually comes from a structural rather than pigment based  
21  
22 163 colouration, we calculated colour variables separately over those 2 regions: 320-490 nm for  
23  
24 164 UV and 491-700 nm yellow-orange (YO) colours. The spectral intensity of the beak spot,  
25  
26 165 mean UV-violet brightness ( $UV_{\text{brightness}}$ ) and mean yellow-orange brightness ( $YO_{\text{brightness}}$ )  
27  
28 166 were calculated by averaging reflectance over wavelengths 320-490 nm and 491-700 nm,  
29  
30 167 respectively (Montgomerie 2006). Hue is a measure of colour appearance (e.g. 'blue',  
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32 168 'yellow', etc.). For the yellow-orange plateau portion of the spectrum,  $YO_{\text{hue}}$  was calculated  
33  
34 169 as the wavelength at which the reflectance was halfway between its maximum and minimum  
35  
36 170 (Keddar et al. 2013). For the UV-violet peak,  $UV_{\text{hue}}$  was calculated as the wavelength of  
37  
38 171 maximum reflectance between 320 and 490 nm. Finally, chroma is a measure of colour  
39  
40 172 purity and was calculated within the region of interest ( $UV_{\text{chroma}}$  and  $YO_{\text{chroma}}$ ) as the  
41  
42 173 difference between maximum and minimum reflectance over the mean reflectance for that  
43  
44 174 particular region (formula S<sub>8</sub>; Hill and McGraw 2006, p. 108). Repeatability of the colour  
45  
46 175 measurements are high (between 0.70 and 0.91) and given in ESM 1. Correlations between  
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48 176 those spectral parameters are presented in Fig. 1a (see also Viblanc et al. 2016). Briefly, the  
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3 177 correlations between UV and YO colour parameters are very low, consistent with the fact  
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5 178 that those colours in the king penguin are produced by two independent mechanisms  
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8 179 (structural for the UV and pigmentary for YO colours; see Dresp et al. 2005). For UV  
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10 180 colours, brightness, hue and chroma parameters were independent and therefore we  
11  
12 181 considered them separately in further analyses. For YO colours however, those parameters  
13  
14 182 were highly correlated. Thus, we chose to focus  $YO_{\text{chroma}}$  for the following reasons. First, it  
15  
16 183 has been argued that the signal with the highest among-individual variance also contains the  
17  
18 184 most information (Dale 2006).  $YO_{\text{chroma}}$  was the parameter the most correlated with both  
19  
20 185 brightness and hue, and also that presenting the highest Coefficient of Variation (Fig. 1b).  
21  
22 186 Second,  $YO_{\text{chroma}}$  has been shown to directly reflect ornament pigment concentration in  
23  
24 187 several species (Saks et al. 2003; McGraw and Gregory 2004).  
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### 189 **Changes in beak spot colouration following the moult**

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34 190 For adult king penguins, beak spots are renewed each year at the end of the period of moult  
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36 191 of the entire plumage (ESM 2). Moulting of feathers and beak spots occur before the start of  
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38 192 the breeding season in November-January and the whole process takes ca. 32 days  
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40 193 (Groscolas and Cherel 1992). To study the maturation of the new beak spot following the  
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42 194 moult process, 25 moulting males were caught shortly before the end of moult and kept  
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44 195 captive in a pen. Birds were checked daily for moult completion, and the colouration of their  
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46 196 beak spot was measured before the moult started and after the moult was completed.  
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48 197 Seventeen birds were kept captive for an extra 2 days, (5 were released because were close to  
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50 198 reaching a critical body mass, i.e. phase 3 of fasting; Groscolas and Robin 2001) and the  
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52 199 colouration of the beak spot was measured a second time. All birds were released and left at  
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3 200 sea to forage. Before being released, birds were identified by marking them on the belly with  
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5 201 a unique letter/number combination using a non-toxic human hair dye (Franck Provost, blue-  
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8 202 black 2.1). The beach was walked every day to search for birds that returned to the colony to  
9  
10 203 start breeding after having being at sea to feed and replenish their body reserves. Eighteen of  
11  
12 204 the 25 followed birds were caught on the beach within a few hours after returning from their  
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14 205 foraging trip and beak measurements were taken a final time. At the end of the moult, 3  
15  
16 206 individuals presented no reflectance signal whatsoever in the UV part of the beak  
17  
18 207 (completely flat spectrum) but a classical YO reflectance spectrum, clearly due to a lack of  
19  
20 208 maturation of the UV component. These 3 individuals were not taken into account in the  
21  
22 209 analyses of UV colour parameters, explaining the variation of the sample size between UV  
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24 210 and YO colour analyses (i.e.  $N = 22$  vs. 25).

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26  
27 211 We studied changes in beak colouration following the moult by specifying beak  
28  
29 212 colour variables (hue, chroma and brightness) as dependent variables in separate Linear  
30  
31 213 Mixed Models (LMMs). The time period of colour measurement was entered as a discrete  
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33 214 ordinal variable with 3 levels: the day the old beak spots were shed (day 0), two days later  
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35 215 (day 2), and the day birds returned from their post-moult foraging trip at sea (courtship). Bird  
36  
37 216 ID was entered as a random effect to account for repeated measurements on individual birds.  
38  
39 217 Correlation between colour parameters before moult and after their post-moult foraging trip  
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41 218 (courtship) were investigate using Spearman's rank test on 16 birds for which we had both  
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43 219 measurements.  
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53 221 **Changes in beak colouration during fasting**  
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3 222 We investigated the effect of fasting on beak spot colouration using either breeding penguins  
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5 223 that were naturally fasting while incubating their egg or of captive penguins that were forced  
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8 224 to fast. We ran three different studies that covered different fasting and duration periods for  
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10 225 this species.

11  
12 226 In a first study performed in 2011-12, we studied 22 males and 22 females during the  
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14 227 third and fourth incubation shift, respectively. We measured beak spots directly in the colony  
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16 228 while birds were incubating their eggs. Birds were measured on their second day of  
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18 229 incubation and again 6 days later (incubation day 8). We waited 2 days before the first  
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20 230 measure to insure that individuals had settled on their eggs, thus avoiding any risk of  
21  
22 231 breeding abandonment. Those birds were all returning from their foraging trip before taking  
23  
24 232 their incubation shift, and thus this study covers changes in beak colouration during the first  
25  
26 233 days of fasting.

27  
28 234 In a second study performed in 2014-15, we investigated changes in beak colouration  
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30 235 of 36 breeding males during their first incubation shift. Beak colouration was measured on  
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32 236 the third day after the start of incubation and again 10 days later, i.e. on day 13 of incubation.  
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34 237 Males do not return at sea to feed between the period of courtship display and their first  
35  
36 238 incubation shift, and thus they had already endured at least 10-days of fasting before our first  
37  
38 239 measurement of colouration. Hence, this study covers changes in beak colouration during the  
39  
40 240 second half of a long (> 20 days) fasting period.

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42 241 Finally, in a third study performed in 2013-14 and 2014-15, we caught 20 males  
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44 242 during courtship on land (10 birds each year) and kept them captive in a pen. These  
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46 243 individuals experienced a forced fasting period (0-24 days) covering the natural fasting  
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48 244 periods of our first (2-8 days) and second (ca. 13-23 days) studies presented above. Such  
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3 245 prolonged fasting periods are well within the natural range of fasting observed in this species.  
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5 246 In these birds, we measured body mass and beak spot colouration every 6 days to investigate  
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8 247 changes in colour as fasting progressed. Upon release, all birds were observed departing to  
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10 248 sea to feed and subsequently seen returning at the colony to breed.

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12 249 Within each experiment, monitored birds were all caught at same day of the same  
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14 250 breeding shift and thus had comparable breeding status. Changes in beak colouration during  
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16 251 fasting were investigated by specifying beak colour variables (hue, chroma and brightness) as  
17  
18 252 dependent variables in separate LMMs. The time period of colour measurement was entered  
19  
20 253 as a discrete ordinal variable (e.g. day 2 and day 8 in the first study). Bird sex was entered as  
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22 254 a fixed factor in the models, and for the first experiment (the only one where both sexes were  
23  
24 255 monitored), we considered the interaction of sex and time. However, as the interaction was  
25  
26 256 not significant, we removed it from the final model. In addition, we accounted for body girth,  
27  
28 257 a proxy to body condition (Viblanco et al. 2012), as a covariate in the models. Again however,  
29  
30 258 as body girth never significantly affected colour parameters, we removed it from the final  
31  
32 259 models. Bird ID was entered as a random effect to account for repeated measurements on  
33  
34 260 individual birds. In the third study, captive birds were measured in two different years (2013-  
35  
36 261 2014 and 2014-2015), and thus we entered the year as random factor to control for potential  
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38 262 year effects on colouration (Keddar, Couchoux, et al. 2015).  
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#### 48 264 **Response of beak colouration to experimental parasite removal**

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50 265 In 2011-2012, we investigated the effects of seabird parasite loads on beak colouration by  
51  
52 266 experimentally removing parasites in an experimental group of 20 breeding pairs using the  
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54 267 anti-parasitic solution Eprinex Pour-On®. This solution is commonly used in cattle and  
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3 268 poultry and known to remove a large spectrum of ecto- and endo-parasites including worms,  
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5 269 lice, ticks, mange mites and grubs) (Shoop et al. 1996). A control group of 20 pairs was  
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8 270 treated with a solution of propylene glycol, which is the solvent used in Eprinex Pour-On®.  
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10 271 We obtained information on changes in beak spot colouration in 27 treated birds (14 males,  
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12 272 13 females) and 33 control birds (16 males, 17 females). To control for possible confounding  
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14 273 effects of breeding timing and localization in the colony on inter-individual variation in beak  
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16 274 spot colouration, we applied our treatments so that treated and control pairs did not differ in  
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18 275 their breeding onset (all were early breeders) or where they were breeding in the colony. The  
19  
20 276 anti-parasitic and control solutions were deposited on the skin of the birds just below the  
21  
22 277 feathers at the base of the neck at the beginning of the first incubation shift and of the third  
23  
24 278 incubation shift in males and at the beginning of the second and fourth incubation shift in  
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26 279 females. The efficiency of the anti-parasitic treatment was controlled by counting tick loads  
27  
28 280 (*Ixodes uriae*) on a small part of the body (the head) of monitored birds. At the start of the  
29  
30 281 treatments (i.e. beginning of shift one of males and shift two of females), there was no  
31  
32 282 difference in the number of ticks on the head of birds treated with the anti-parasitic versus  
33  
34 283 sham solutions (mean  $\pm$  s.e. = 2.26 ticks  $\pm$  0.62 versus 1.39 ticks  $\pm$  0.59; Wilcoxon's test:  $chi^2$   
35  
36 284 = 0.21,  $df = 1$ ,  $P = 0.64$ ). The Eprinex Pour-On® solution was efficient at removing parasites  
37  
38 285 as reflected by lower tick loads after treatment on the head of birds treated with the anti-  
39  
40 286 parasitic versus sham solutions (0.05 ticks  $\pm$  0.64 versus 3.15 ticks  $\pm$  0.62; Wilcoxon's test:  
41  
42 287  $chi^2 = 27.23$ ,  $df = 1$ ,  $P < 0.001$ ). Effects of our treatments on changes in beak spot colouration  
43  
44 288 were measured during shift 3 and 4 of males and females, respectively, by taking a first  
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46 289 measure of beak colouration on the second day after the start of incubation and again 6 days  
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55 290 later, i.e. on day 8 of incubation.  
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3 291 Changes in beak colouration in response to our treatments were investigated by  
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5 292 specifying beak colour variables (hue, chroma and brightness) as dependent variables in  
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7  
8 293 separate LMMs. The effect of the experimental anti-parasitic treatment on beak colouration  
9  
10 294 was tested from the significance of the interaction between treatment (anti-parasitic vs. sham)  
11  
12 295 and time period (day 3 vs. day 8) in the model. Sex was added as a fixed factor in the model  
13  
14 296 to test for potential sex differences in beak colouration variation and its interaction with  
15  
16 297 treatment was also considered. Again, body girth was added as a covariate in the model and  
17  
18 298 its interaction with treatment and period considered, but as it never showed any significant  
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20 299 effect on colour parameter, it was removed from the final models. Bird ID was entered as a  
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22 300 random effect to account for repeated measurements on individual birds.  
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### 302 **Statistics**

303 Statistical analyses were run with R v.3.1.1 (R development core team). F-statistics for fixed  
304 effects (tests of differences from zero), the total number of observations ( $n$ ) and  
305 corresponding number of individuals ( $N$ ) are given. Effects were considered significant for  $P$   
306  $< 0.05$ . When appropriate, significant differences between groups were assessed using  
307 Tukey's Honest Significant Difference (HSD) tests for least square means. We insured  
308 residuals followed a normal distribution using qqplots (opposing theoretical Quantiles to  
309 Sample Quantiles).

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### 311 **Ethical statement**

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



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3 314 Institute and comply with the current laws of France. Authorizations to enter the  
4  
5 315 breeding colony and handle the birds were provided by the “Terres Australes et  
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7 316 Antarctiques Françaises” (permit n°2010-65 issued on the 3<sup>rd</sup> of September 2010,  
8  
9 317 n°2011-96 issued on the 14<sup>th</sup> of October 2011, n°2012-116 issued on the 29<sup>th</sup> of  
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11 318 October 2012, n°2013-72 issued on the 29<sup>th</sup> of October 2013 and n°2014-127 issued on  
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13 319 the 15<sup>th</sup> of October 2014).  
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## 20 321 RESULTS

### 22 322 Changes in beak spot colouration following the moult

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25 323 Birds showed pronounced changes in the colouration of their beak spot following the  
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27 324 moult. The new beak spot appeared particularly pinkish and changed from day to day (Fig.  
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29 325 2).  $UV_{\text{brightness}}$  of the new beak decreased by 14% within the first 2 days of shedding the old  
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31 326 beak spot, and continued to decrease by another 23% during the post-moult foraging trip at  
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33 327 sea (LMM;  $F = 79.21$ ,  $df = 2$ ,  $P < 0.001$ ,  $n = 55$ ,  $N = 22$ ). Freshly moulted birds exhibited a  
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35 328 rapid decrease in the  $UV_{\text{hue}}$  of their new beak spot (by 20.7 nm on average) within the first 2  
36  
37 329 days of shedding the old beak spot (Fig. 2).  $UV_{\text{hue}}$  continued to decrease during the post-  
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39 330 moult foraging trip (another 10.7 nm on average), albeit less substantially ( $F = 30.20$ ,  $df = 2$ ,  
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41 331  $P < 0.001$ ,  $n = 55$ ,  $N = 22$ ). We observed increases both in  $UV_{\text{chroma}}$  (+19% within 2 days  
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43 332 post-moult and another 23% by the time the birds returned for courtship;  $F = 51.04$ ,  $df = 2$ ,  $P$   
44  
45 333  $< 0.001$ ,  $n = 55$ ,  $N = 22$ ) and  $YO_{\text{chroma}}$  (+24% within 2 days post-moult, and another 13% by  
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47 334 courtship;  $F = 23.88$ ,  $df = 2$ ,  $P < 0.001$ ,  $n = 60$ ,  $N = 25$ ).  
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53 335 When compared colour parameters before moult and after birds came back from their  
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55 336 post-moult foraging trip,  $UV_{\text{hue}}$  was significantly correlated (Spearman's rank correlation;  $\rho$   
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337 = 0.64,  $S = 246.7$ ,  $P = 0.008$ ,  $n = 30$ ,  $N = 15$ , Fig. 3), other parameters remained uncorrelated  
338 ( $-0.36 < rho < 0.38$ ,  $422 < S < 992$ ,  $0.148 < P < 0.51$ ,  $n = 30$ ,  $N = 15$ ).

339

#### 340 **Changes in beak spot colouration during fasting**

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

348 In captive birds that fasted up to a similar body mass of that naturally occurring at  
349 partner relief (24 days), fasting duration did not affect UV beak colouration (brightness, hue  
350 or chroma) (LMMs:  $0.92 < F < 2.04$ ,  $df = 4$ ,  $0.10 < P < 0.46$ ,  $n = 91$ ,  $N = 20$ ). In contrast,  
351  $YO_{\text{chroma}}$  decreased ( $F = 4.23$ ,  $df = 4$ ,  $P < 0.05$ ) with increased fasting (Fig. 5), and showed a  
352 strong significant decrease at the end of the fasting period (Tukey's HSD:  $-3.63 < Z < -2.77$ ,  
353  $0.003 < P < 0.044$ , ESM 3).

354

#### 355 **Response of beak colouration to experimental parasite removal**

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

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3 360  $0.80 < P < 0.99$ ,  $n = 120$ ,  $N = 60$ ). Bird sex, whether considered independently or in  
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5 361 interaction with treatments and/or time period, never significantly affected beak colouration  
6  
7 362 ( $0.12 < P < 0.96$   $n = 120$ ,  $N = 60$ ). The effect of sex was removed from the final models  
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9 363 presented in Table 1.  
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## 14 365 **DISCUSSION**

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17 366 Dynamic ornamental signals can provide continuous information on individual condition, and  
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19 367 are expected to be under strong sexual and social selection (e.g. Velando et al. 2006). In  
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21 368 birds, beak colouration has been proposed to function as a dynamic ornament (Faivre,  
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23 369 Grégoire, et al. 2003; Navarro et al. 2010; Rosenthal et al. 2012) as indeed appears to be the  
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25 370 case in our species. King penguins showed a rapid maturation of beak colouration following  
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27 371 the moult and we found dynamic changes in colouration in response to changes in individual  
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29 372 condition (fasting status and parasite load) in breeding birds over the scale of a single  
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31 373 breeding season.  
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### 38 375 **Changes in beak spot colouration following the moult**

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40 376 Although studies on avian moult are numerous, there is virtually no information on moulted  
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42 377 structures other than feathers (King and Murphy 1990). Horn, claw and beak material are  
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44 378 thought to grow continuously in response to wear, but king penguins appear to be an  
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46 379 exception in that the entire horny material of their beak spot is shed every year at the end of  
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48 380 the moult, while the rest of the black beak horn is not. Thus, beak spots are replaced each  
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50 381 time these long-lived seabirds breed, most of the time attracting a new breeding partner  
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52 382 (Bried et al. 1999; Olsson 1998). After the moult, beak spots colouration becomes less bright  
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3 383 changing to a deeper (decrease in hue) and purer UV colour (Fig. 2, ESM4). As previously  
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5 384 mentioned, the UV colouration of penguin beaks is structural, resulting from the reflection of  
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8 385 stacks of elongated lamellae (multiple layers of doubly folded membranes) in the horny layer  
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10 386 of the beak (Dresp and Langley 2006). The distance (in nm) separating those double-folds is  
11  
12 387 responsible for  $UV_{hue}$ , i.e. the lattice dimension of the photonic crystals (Dresp and Langley  
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14 388 2006). This photonic property may be explained by Bragg's law, with  $\lambda_{max} = n2d \sin\theta$ ,  
15  
16 389 where  $\lambda_{max}$  is the peak wavelength of reflected light,  $n$  is the average refractive index of the  
17  
18 390 tissue,  $d$  is the separation of the layers (lattice dimension), and  $\theta$  is the angle of incidence of  
19  
20 391 the light (Bragg 1915). Thus, our results suggest that as the beak matures, there is a decrease  
21  
22 392 in the distance between the doubly folded membrane structures that compose the upper-layer  
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24 393 of the beak. Simultaneously, although the magnitude of change is weaker, the yellow-orange  
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26 394 colour of the beak also becomes purer as chroma increases. These latter colour changes occur  
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28 395 as the bird prepares to mate, over a period of 13-20 days and are likely explained by the  
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30 396 deposition of carotenoid pigments in the deeper layers of beak spots (higher chroma), as is  
31  
32 397 the case in many bird species (Saks et al. 2003; McGraw and Gregory 2004). Interestingly,  
33  
34 398 comparing colours before and at courtship for the same birds,  $UV_{hue}$  appeared strongly  
35  
36 399 correlated (Fig. 3). It suggest that  $UV_{hue}$  (distance between doubly-folded membranes) is  
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38 400 determined largely genetically, but some scope for variation does exist (i.e. time since the last  
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40 401 moult, time spent at sea during the past year, time spend preening, etc.  
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### 51 **Beak spot colouration and fasting**

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53 404 Nutritional status and body condition are important information that may be used by  
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55 405 conspecifics both in reproductive and social contexts. In the Alpine swift and the European  
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3 406 starling for instance, parents adapt their feeding effort to the UV colouration of chick skin,  
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5 407 which is positively correlated with body mass and structural size, and is used by parents to  
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8 408 adapt their feeding behaviour to the intensity of the reflected colour of their chicks (Bize et  
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10 409 al. 2006). In the same way, in blue-footed boobies, rapid experimental changes in male foot  
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12 410 integument colouration (reflecting nutritional status) elicited rapid adjustments in female  
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14 411 reproductive strategies, i.e. facilitation of brood reduction by laying smaller eggs compared  
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16 412 to controls (Velando et al. 2006). In our study, male beak colouration appeared to reflect  
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18 413 changes in nutritional status. Over 24 fasting days, captive males showed a progressive  
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20 414 decline in  $YO_{\text{chroma}}$  but no change in UV colouration. However, when fasting was prolonged  
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22 415 for even longer periods in colonial conditions (over >24 days including 11-15 days of  
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24 416 courtship followed by 13 days of incubation; Fig 4),  $UV_{\text{brightness}}$  appeared to decrease. Those  
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26 417 results suggests that UV and YO colouration may signal fasting status on different time-  
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28 418 scales. A progressive decrease in  $YO_{\text{chroma}}$  in fasting birds may reflect a re-allocation of beak  
29  
30 419 pigments to antioxidant defences, in line with the hypothesis of costly signalization by  
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32 420 (limited) carotenoid-dependent structures (e.g. in birds: Alonso-Alvarez et al. 2004; in fish:  
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34 421 Pike et al. 2010; but see Cote et al. 2010 in reptiles). This suggestion is consistent with our  
35  
36 422 recent data suggesting that plasmatic anti-oxidant defences indeed increase over the course of  
37  
38 423 fasting (QS, FC, VAV, AS, JPR and PB; *unpublished data*). Yellow-orange colours are often  
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40 424 produced by exogenous carotenoid pigments, which are acquired from the diet and may  
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42 425 reflect yearly environmental forage conditions (Linville and Breitwisch 1997; McGraw et al.  
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44 426 2009; Slagsvold and Lifjeld 2009). In king penguins, similar mechanisms might explain  
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46 427 variation in YO colour production between years (i.e. higher YO colour in years of high  
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48 428 resource availability; Keddar, Couchoux, et al. 2015). Decreases in  $UV_{\text{brightness}}$  only at  
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3 429 advanced fasting stages may suggest a cost for UV maintenance and the inability to maintain  
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5 430 high UV reflectance when energy is critically limiting. For instance, preening and associated  
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8 431 comfort behaviour (keeping the beak clean) is generally costly in birds (Walther and Clayton  
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10 432 2004), including king penguins (Viblanco et al. 2011), and reducing those behaviour may  
11  
12 433 allow substantial savings at advanced stages of fasting. Whether such behavioural changes  
13  
14 434 linked to fasting status might indirectly affect the maintenance of beak colouration remains to  
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16  
17 435 be tested. Furthermore, as captive birds and free-living breeders also differed in their social  
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19 436 environmental and breeding status, we cannot exclude that such differences also affected  
20  
21 437 changes in beak colouration differently between the two groups. Further investigations are  
22  
23 438 needed to clarify how beak spot dynamics are conditioned by the rate of nutrient reserves  
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25 439 mobilization and availability of dietary antioxidants such as carotenoids by following  
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27 440 plasmatic availability concomitantly to changes in colouration.  
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#### 33 34 442 **Beak spot colouration and parasites**

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36 443 Parasites drain resources (including pigments and nutrients) from their hosts, which might  
37  
38 444 otherwise be partly allocated to producing ornaments. Furthermore, in fighting parasites,  
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40 445 hosts also mount immune responses that divert resources from other functions such as  
41  
42 446 ornamentation (Rosenthal et al. 2012; Velando et al. 2014). Regardless of the underlying  
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44 447 mechanism, parasitism should affect ornamental signals (e.g., parasite-mediated sexual  
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46 448 selection; Hamilton and Zuk 1982). Consistent with this hypothesis, we found that removing  
47  
48 449 parasites from males and females during incubation produced significant changes in the UV  
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50 450 component of beak colouration. While not influencing YO colours, removing parasites  
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53 451 resulted in beak spots of higher  $UV_{\text{brightness}}$ , higher  $UV_{\text{hue}}$  and lower  $UV_{\text{saturation}}$  (Fig. 6). Birds  
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3 452 relieved of parasites should have a less stimulated immune system, and may therefore invest  
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5 453 more into beak colouration. Accordingly, several studies in birds have highlighted strong  
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8 454 links between UV ornamentation and parasite load (Hörak et al. 2001; Mougeot et al. 2010)  
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10 455 or in a broader context immune-competence (Griggio et al. 2010; Peters et al. 2008).

11  
12 456 Here again, the independent changes we observe in UV or YO colouration support  
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14 457 previous findings that in king penguins, UV and YO colours of the beak are produced by  
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16 458 distinctly mechanisms (Dresp and Langley 2006), and appear to change over the course of  
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18 459 the breeding season. Indeed, YO carotenoid-based colouration is produced by pigments  
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20 460 embedded in the deeper beak layers and was relatively unaffected in our anti-parasite  
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22 461 treatment. In contrast, changes in beak UV suggest structural modifications. Since UV  
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24 462 reflectance depends on the thickness of the upper beak layer composed of the doubly folded  
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26 463 membrane structures, which result from the differentiation of basal cells into dead keratin (as  
27  
28 464 is the case for skin renewal) (Dresp and Langley 2006), an increase in UV reflectance  
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30 465 following parasite removal suggests either an increase in cell division or a reorganisation of  
31  
32 466 those structures. However, whether the thickness of the upper beak layer can be increased  
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34 467 after the moult (i.e. complete renewal of the structure), or whether it can be remodelled is  
35  
36 468 currently unknown and requires further investigations. Moreover, ectoparasites are known as  
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38 469 vectors of disease such as ticks with Lyme Disease (Gauthier-Clerc et al. 1999), which would  
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40 470 not be cured with our anti-parasitic treatment and could limit the effect of our experimental  
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42 471 treatment. Whereas other proximal mechanisms are likely, our results point to parasitism as  
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44 472 an important influence on structural based UV colour signalisation in the king penguin.  
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55 474 **Concluding remarks**  
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3 475 Beak colouration of king penguins can be highly flexible, with both components of beak  
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5 476 colouration (UV and YO) modified independently. Both hue and chroma of the UV, and  
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7 477 chroma of the YO of beak spot appear to have a maturation process, with an associated  
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9 478 decline in beak spot brightness that continues through pre-breeding moult, subsequent  
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11 479 feeding at sea, return to the breeding grounds, and mating. Importantly, due to its dynamicity,  
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13 480 beak colouration may serve as an important signal of short-term changes in individual  
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15 481 condition over the course of a single breeding season. This supports the idea that the  
16  
17 482 information conveyed by sexual ornaments is not restricted to the single time period when  
18  
19 483 mate choice occurs (e.g. Velando et al. 2006; Ardia et al. 2010).

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22 484 Previous studies have found that higher beak  $UV_{\text{brightness}}$  is associated with higher  
23  
24 485 mating prospects (Nolan et al. 2010) and different body condition in males and females  
25  
26 486 (Dobson et al. 2008; Viblanc et al. 2016), and higher beak  $UV_{\text{hue}}$  is negatively related with  
27  
28 487 hormonal stress responses and oxidative damages in female (Viblanc et al. 2016). In line with  
29  
30 488 these studies, our current results highlighted positive associations between  $UV_{\text{brightness}}$  and  
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32 489  $YO_{\text{chroma}}$  and fasting, when condition decrease colour decrease. In the contrary, when during  
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34 490 breeding parasite are removed the condition of the birds increase as well as  $UV_{\text{brightness}}$ . The  
35  
36 491 next step is to focus on how such variations might be perceived by the mate at the time it  
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38 492 takes over egg or chick-guarding duties, how beak colouration may help parents coordinate  
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40 493 their efforts throughout the breeding season, and how dynamic changes in beak colouration  
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42 494 may be perceived by social conspecifics.  
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10 661 **TABLES**  
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15 663 **Table 1. Mixed model estimates ( $\pm$  SE) for the effects of an anti-parasitic treatment on**  
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17 664 **beak colouration in breeding king penguins (*Aptenodytes patagonicus*).** The control [C]  
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19 665 level for the treatment factor is tested against the treatment level [T]. The time period [post-  
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21 666 treatment] is tested against the level [pre-treatment]. Bird ID were entered as random factors  
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23 667 in the model to account for repeated measures on the individual. Values are significant for  $P$   
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25 668  $< 0.05$ . Note the significant interaction terms revealing different treatment effects on pre- and  
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27 669 post-treatment measures of treated vs. control birds.  $n = 120$  observations,  $N = 60$  birds.  
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34 671 **FIGURE CAPTIONS**  
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39 673 **Fig 1. a) Pairwise correlation plots between the different colour parameters in king**  
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41 674 **penguin beak spots.** Spearman correlation coefficients are provided and the distribution of  
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43 675 the each colour parameter is presented. Note that UV and YO colour parameters are weakly  
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45 676 correlated b) Coefficient of variation of the different YO colour parameters. We compiled  
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47 677 first measurements of the 181 individuals (36 where in courtship and 145 at the beginning of  
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49 678 their breeding shift).  
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55 680 **Fig. 2. Changes in the beak colouration of adult king penguins (*Aptenodytes***  
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3 681 *patagonicus*) following the moult. Average changes in raw spectral data over all birds are  
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5 682 presented for illustrative purposes. Beak colour was measured on the day the old beak spot  
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7 683 was shed (day 0), 2 days later (day 2), and after birds returned from their post-moult foraging  
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9 684 trip for breeding (courtship). Changes in brightness, hue and chroma were assessed using  
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11 685 LMMs, with bird ID specified as a random variable. Least-Square means  $\pm$  SE are presented.  
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13 686 Values not sharing a common letter are significantly different for  $P < 0.05$ . The sample sizes  
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15 687 are given below the means.  
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22 689 **Fig. 3 Correlation between  $UV_{\text{hue}}$  before the moult and after the post-moult foraging**  
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24 690 **trip at the time the birds come for courtship.** Correlation were assessed using Spearman's  
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26 691 rank correlation test ( $\rho = 0.64$ ;  $S = 246.7$ ;  $P = 0.008$ ).  
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32 693 **Fig. 4 Changes in beak  $UV_{\text{brightness}}$  and  $YO_{\text{chroma}}$  for 36 male king penguins (*Aptenodytes***  
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34 694 ***patagonicus*) that were measured on day 3 of their first incubation shift and on day 13**  
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36 695 **after having experienced a 10-day fast while breeding in the colony.** Changes in  
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38 696 brightness were assessed using LMMs, with bird ID specified as a random variable. Least-  
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40 697 Square (LS) means  $\pm$  SE are presented. Values not sharing a common letter are significantly  
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42 698 different for  $P < 0.05$ . The sample sizes are given below the means.  
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48 700 **Fig. 5 Changes in yellow-orange beak chroma ( $YO_{\text{chroma}}$ ) in 20 males that endured a**  
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50 701 **prolonged fast in captivity.** Changes in chroma were assessed using LMMs, with bird ID  
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52 702 and year specified as a random variable.  
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704 **Fig. 6. Interactive effects of an experimental anti-parasitic treatment on the beak**  
 705 **colouration of adult kings penguins (*Aptenodytes patagonicus*) freely incubating in the**  
 706 **colony.** Changes in brightness, hue and chroma were assessed using LMMs, with bird ID  
 707 specified as a random variable, the treatment (treated vs. sham), the time period (pre-  
 708 treatment vs. post-treatment) and the interaction between those factors specified as  
 709 independent variables. Least-Square (LS) means are presented. Values not sharing a common  
 710 letter are significantly different for  $P < 0.05$ .

712 **Table 1**

	<b>Term</b>	<b>Estimate <math>\pm</math> SE</b>	<b>t Ratio</b>	<b>Prob &gt;  t </b>
<b>UV<sub>brightness</sub></b>	Intercept	18.56 $\pm$ 0.57	32.99	<0.001*
	Period[Post-treatment]	-0.52 $\pm$ 0.66	-0.78	0.437
	Treatment [T]	-0.23 $\pm$ 0.84	-0.27	0.787
	Period*Treatment	4.68 $\pm$ 0.98	4.79	<0.001*
<b>UV<sub>hue</sub></b>	Intercept	388.65 $\pm$ 1.51	256.57	<0.001*
	Period[Post-treatment]	0.12 $\pm$ 1.10	0.11	0.915
	Treatment [T]	-1.34 $\pm$ 2.26	-0.61	0.54
	Period*Treatment	4.15 $\pm$ 1.62	2.56	<0.001*
<b>UV<sub>chroma</sub></b>	Intercept	1.33 $\pm$ 0.03	46.21	<0.001*
	Period[Post-treatment]	-0.02 $\pm$ 0.04	-0.43	0.666
	Treatment [T]	0.08 $\pm$ 0.04	1.87	0.064
	Period*Treatment	-0.12 $\pm$ 0.05	-2.20	0.032*
<b>YO<sub>chroma</sub></b>	Intercept	1.16 $\pm$ 0.04	28.27	<0.001*
	Period[Post-treatment]	-0.01 $\pm$ 0.06	-0.13	0.898
	Treatment [T]	0.13 $\pm$ 0.06	2.16	0.032*
	Period*Treatment	-0.10 $\pm$ 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.

For Review Only

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