

1 Title:

2 Sexual skin color contains information about the timing of the fertile phase in free-ranging
3 rhesus macaques

4

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23

24 ABSTRACT

25

26 Females of several primate species undergo cyclical changes of their sexual skin, namely the
27 development of a swelling and/or a change in color. The relationship between within cycle
28 probability of fertility and the size of sexual swellings is well established, but the only study to
29 combine an objective measure of color with endocrinological data found no evidence that
30 swelling color contains such information. To evaluate the role of female skin color in the
31 context of sexual signaling further, we investigate whether changes in sexual skin color contain
32 information about the timing of the fertile phase in rhesus macaques, a species in which adult
33 females do not develop sexual swellings, but do express visually detectable changes in the skin
34 color of the face and hindquarters. Using an objective and quantitative measure of color, along
35 with detailed data on fecal progesterone and estrogen metabolite levels collected from 8
36 females of the Cayo Santiago colony, we show that the ratio of red to green (R/G) for facial and
37 hindquarter skin significantly varies throughout the ovarian cycle. In addition, facial skin R/G is
38 significantly higher during the 5-day fertile phase compared to the 5-day periods immediately
39 preceding or following this time, but no such pattern is found in hindquarter R/G. This suggests
40 that skin color change in female rhesus macaques may potentially signal information about the
41 within cycle probability of fertility to male receivers, but that only facial skin color may signal
42 reliable information about its timing.

43

44 KEYWORDS

45 Sexual skin, color, fertile phase, fecal steroids, *Macaca mulatta*

46

47 INTRODUCTION

48

49 In several primate species, there is variation over the ovarian cycle in the sexual skin of females,
50 namely an increase in size ('sexual swelling') and/or change in color (Dixson 1983; Nunn 1999;
51 Zinner et al. 2004). Among cercopithecines, the ancestral state appears to include changes in
52 both the size and color of the skin of the anogenital region and its surrounding areas, and a lack
53 of these traits is most likely due to secondary loss (Dixson 1983; 1998; Nunn 1999). Members of
54 this subfamily who express changes in skin color, but who do not have sexual swellings, may
55 therefore represent an intermediate evolutionary stage to a complete loss of sexual skin
56 changes (Sillén-Tullberg and Møller 1993; Dixson 1998). All such species express skin color
57 changes in the anogenital region (e.g. vervets, *Cercopithecus aethiops*, and patas monkeys,
58 *Erythrocebus patas*; Dixson 1983; 1998), while some also express color change in ventral
59 anatomical areas such as the chest and abdomen (gelada baboons, *Theropithecus gelada*:
60 Matthews 1956; Alvarez 1973), as well as the face (e.g., rhesus macaques, *Macaca mulatta*:
61 Zuckerman et al. 1938; Baulu 1976; Japanese macaques, *M. fuscata*: Fujita et al. 2004).

62

63 Given that changes in the sexual skin of cercopithecines occur over the ovarian cycle, they may
64 contain information regarding the within cycle likelihood of conception. Indeed, a relationship
65 between the size of sexual swellings and the timing of the fertile phase is well established (e.g.,
66 *Macaca nigra*: Thomson et al. 1992; *M. tonkeana*: Aujard et al. 1998; *Pan troglodytes*: Deschner
67 et al. 2003; *M. fascicularis*: Engelhardt et al. 2005; *M. sylvanus*: Möhle et al. 2005; *Hylobates*
68 *lar*: Barelli et al. 2007; *Papio cynocephalus*: Geschiere et al. 2007; *Papio anubis*: Higham et al.

69 2008). In contrast, few studies have investigated the potential link between female fertility and
70 red skin coloration (Bradley and Mundy 2008), even though catarrhine species are trichromats
71 and thus perceive red (reviewed in: Surridge et al. 2003; Waitt and Buchanan-Smith 2006), and
72 red skin coloration has been proposed to be an important socio-sexual signal in these species
73 (Changizi et al. 2006; Fernandez and Morris 2007). Of those studies undertaken, most lacked an
74 objective means to quantify color (Czaja et al. 1977; Gauthier 1999; Fujita et al. 2004),
75 endocrine information to determine the timing of the fertile phase (Setchell et al. 2006), or
76 both of these elements (Matthews 1956; Alvarez 1973; Baulu 1976). The only study to date to
77 combine an objective measure of color with endocrinological data found no evidence that
78 anogenital skin color contains precise information regarding the timing of the fertile phase in
79 olive baboons (*P. anubis*), a species with a prominent sexual swelling (Higham et al. 2008). To
80 our knowledge, no comparable analysis has been carried out on a species lacking a prominent
81 swelling. In order to evaluate the role of female skin color in the context of sexual signaling
82 more fully, data for more species based on objective measures of color and detailed hormone
83 profiles are therefore required, with special attention paid to species which do not have sexual
84 swellings.

85

86 Adult female rhesus macaques do not exhibit sexual swellings, but do express changes in the
87 skin color of the face and hindquarters (i.e. the anogenitals, legs, thighs, and base of the tail)
88 which are very pronounced, ranging from pale pink to deep red, and clearly visible to human
89 observers (Zuckerman et al. 1938; Carpenter 1942; Cleveland et al. 1943; Bernstein 1963; Baulu
90 1976; Czaja et al. 1977). There is some evidence to suggest skin color change in rhesus

91 macaques may contain information about when females are most likely to be fertile during the
92 ovarian cycle. For example, estrogen, in addition to its reproductive role, regulates variation in
93 blood flow directly under the skin in this species, which in turn causes changes to skin redness
94 (Rhodes et al. 1997; reviewed in Dixson 1998). Moreover, male rhesus macaques are more
95 attracted to redder skin as shown by experiments that recorded gaze durations toward images
96 of adult females (Waitt et al. 2006; Gerald et al. 2009). The most direct evidence, however,
97 comes from studies by Baulu (1976) and Czaja et al. (1977). Using subjective observer ratings of
98 color intensity, and reproductive assessment via reproductive hormones (Czaja et al. 1977) or
99 visual inspection of menstruation (Baulu 1976), these authors found that the hindquarters of
100 captive females are reddest at mid-cycle (i.e. when females are more likely to be fertile). While
101 these results are promising, in order to understand the relationship between skin color and the
102 timing of the fertile phase in rhesus macaques, studies in which both reproductive status and
103 color are quantified objectively are required.

104

105 In this study, we combine an objective measure of color with detailed hormonal data to
106 examine whether facial and hindquarter skin color of free-ranging adult female rhesus
107 macaques varies over the course of the ovarian cycle in such a way as to reveal information
108 about the timing of the fertile phase. This constitutes the first study of its type in nonhuman
109 primates to investigate the potential role of skin color as a sexual signal in a species without a
110 sexual swelling.

111

112 METHODS

113

114 Study site and subjects

115

116 We studied free-ranging rhesus macaques on Cayo Santiago (Caribbean Primate Research
117 Center, Puerto Rico). Data were collected during the peak of the mating season from April 22nd
118 to July 12th, 2007. At the time of study, our focal group (Group 'V') comprised 22 adult females
119 (≥ 7 years old), 9 nulliparous females (3-5 years old) and 15-20 sexually active males (≥ 4 years
120 old). Data presented in this paper are from 8 parous adult females (average age: 9.3 years,
121 range: 6-17) for which both sufficient fecal samples for assessment of the ovarian cycle, and
122 skin color data, were available. We analyzed 10 ovarian cycles for the face and 8 for the
123 hindquarters. Of the 10 cycles used, 5 were conceptive (as indicated by maintenance of
124 elevated PdG levels for more than 4 weeks and/or occurrence of birth).

125

126 Assessment of skin coloration

127

128 We used digital images of subjects' faces and hindquarters to quantify color. Images were
129 captured using a Canon EOS Digital Rebel XTi camera with a 10.1 megapixel CMOS sensor and
130 an EF28-135mm f/3.5-5.6 IS USM lens. Photographs were taken in RAW format and converted
131 to 16-bit TIFF files for analysis. We attempted to capture all images straight on (i.e. directly
132 facing the camera). Images were captured approximately 1-3 meters from subjects with the
133 flash disabled and with the shutter speed and aperture size determined automatically by the
134 camera. Images were collected between 7:30AM and 10:30AM, a time period leading up to and

135 following the distribution of commercial food by CPRC employees and characterized by feeding
136 behavior and general activity (e.g. traveling, vigilance). Images of subjects in locations that were
137 unevenly or heavily shaded, as well as those in full sunlight, were avoided.

138
139 Immediately following the capture of an image, a second photograph was taken of a color
140 rendition chart (GretagMacbeth ColorChecker, hereafter 'color chart'). Color charts were placed
141 in the same location as the subject, and were photographed under the same lighting conditions
142 using the same shutter speed and aperture size as for the subject image (Higham 2006;
143 Bergman and Beehner 2008; Higham et al. 2008). The color chart consists of 24 colored squares
144 of known and varying reflectance. Following Bergman and Beehner (2008), we adjusted subject
145 images according to the known values of their corresponding color charts using the inCamera
146 plug-in (Pictocolor Corporation, v. 4.0.1) for Adobe Photoshop (CS2, 9.0.1). This technique
147 allows comparisons of color data between images captured under different lighting conditions
148 and with different camera settings.

149
150 To verify whether our method measures color accurately, we tested our outputs for a linear
151 relationship to light intensity and determined whether the reflectance values of the 3 color
152 channels were equal (Stevens et al. 2007; Stevens et al. this issue). To achieve this, we
153 measured the red, green and blue (R, G and B) reflectance values for each of the 6 gray colored
154 squares of 10 adjusted color chart images. Linear regressions of the measured R, G and B values
155 and the known reflectance values of the grey squares yielded an R^2 of 1.0 for all 3 color
156 channels. The absolute difference between measured reflectance values of R, G and B was in

157 the range of 0-2 (out of a maximum possible difference of 255) with a mean (+/- SD) difference
158 of 0.52 ± 0.64 . Based on these findings, we concluded our method measures color accurately.

159
160 Color was measured in 10x10 pixel areas from 3 pairs of points on the face, and from 1 pair of
161 points on the hindquarters in pre-defined locations to ensure consistency between individuals
162 (Figure 1). One pair of points on the face was measured in the middle of the canthal eye region
163 (i.e. the outer corner of the eye; see Figure 1). A second pair of points was measured in the
164 upper cheek region; we selected this point to lie midway along an imaginary line extending
165 horizontally from the hairline to another line, which was extended vertically from the inner
166 corner of the eye to the outer edge of the nostril. We chose the third pair of points to lie
167 midway along a line extending horizontally from the outer edge of the nostril to the hairline.
168 For the hindquarters, we measured color from 1 point directly underneath each of the ischial
169 callosities. These points were all easily identified in straight on and slightly angled images.
170 When a point could not be selected, either because it was obstructed by an object (e.g. a tree
171 branch) or because of the angle of the image, the point was discarded. We determined mean R,
172 G and B values for each point (Jasc Paint Shop Pro 7) and from these calculated mean values for
173 the face and hindquarters. Following Bergman and Beehner (2008), we used the ratio of R to G
174 (hereafter, 'R/G') to assess changes in skin color.

175
176 Assessment of the ovarian cycle and definition of the fertile phase

177

178 We collected a median of 31 fecal samples per focal female during the period of image
179 collection (range: 14-42), with samples collected on average every 2.7 days (range: 1.9-5.8). We
180 collected samples directly after defecation and discarded those that were contaminated with
181 urine. We homogenized fecal boluses and placed 0.5-2 g in individual polypropylene tubes.
182 Samples were kept on ice until they were returned to the field station at the end of the
183 observation day where they were stored at -20°C. Samples were shipped on dry ice to the
184 German Primate Center for hormone analysis.

185

186 Prior to hormone analysis, fecal samples were lyophilized and pulverized (Heistermann et al.
187 1993) and an aliquot of the fecal powder was extracted with 3 ml of 80% methanol in water
188 (Heistermann et al. 1995). The sample extracts were centrifuged (3000 rpm, 5 min) and
189 supernatants were stored at -20°C until assay. Fecal extracts were measured for progesterone
190 and estrogen metabolites using microtiterplate enzymeimmunoassay (EIA) for pregnanediol
191 glucuronide (PdG) and estrogen conjugates (E1C). These assays are described in detail by
192 Heistermann et al. (1995), and have both been successfully used for monitoring female
193 reproductive status and the timing of ovulation in macaque species (Shideler et al. 1993; Fujita
194 et al. 2001; Heistermann et al. 2001). As shown in Figure 2, both assays were highly successful
195 in yielding the typical patterns of estrogen and progesterone during the ovarian cycle in rhesus
196 macaques, from which timing of ovulation and the fertile phase could be reliably deduced (see
197 below). Sensitivity of the assays at 90% binding was 12.5pg for PdG and 1.0pg for E1C. Inter-
198 assay coefficients of variation determined from quality controls were 10.6% (high, N=37) and
199 14.9% (low, N=37) for PdG, and 11.4% (high, N=27) and 14.6% (low, N=27) for E1C. Intra-assay

200 coefficients of variation were 7.2% (high, N=16) and 9.4% (low, N=16) for PdG and 5.3% (high,
201 N=16) and 7.7% (low, N=16) for E1C.

202
203 Fecal progesterone metabolite profiles were used to determine the dates when ovulation most
204 likely occurred (the 'ovulation window'). Ovulation was considered to have occurred when PdG
205 concentrations rose above a threshold of the mean plus 2 standard deviations of 3 to 5
206 preceding baseline values, and maintained at this level for at least 3 consecutive samples
207 (Jeffcoate 1983; Heistermann et al. 2001). On the basis of a time lag of 24-56h in the excretion
208 of reproductive hormone metabolites in the feces of macaques (Shideler et al. 1993) and to
209 account for life span of the oocyte (France 1981; Deschner et al. 2003; Higham et al. 2008), we
210 defined the most likely days of ovulation as days -2/-3 relative to the defined PdG rise
211 (Heistermann et al. 2001; Engelhardt et al. 2004; Brauch et al. 2007) and set the last day of this
212 ovulation window as day 0 (Figure 2). Following Bosu et al. (1973), we set the length of the
213 ovarian cycle at 28 days (Figure 2). The fertile phase was defined as a 5 day period including the
214 2 day ovulation window and the 3 days preceding it to account for sperm life span in the female
215 tract (Behboodi et al. 1991; Wilcox et al. 1995). The 5 days preceding and the 5 days following
216 the fertile phase were referred to as pre- and post-fertile phases (Figure 2).

217

218 Data analysis and statistics

219

220 Only cycles for which the frequency of fecal sample collection during the periovulatory period
221 allowed us to estimate the fertile phase reasonably reliably (i.e. a maximum of a 3-day gap

222 between the day of the PdG rise and the previous sample; median: 2, range: 0-3) and for which
223 at least one picture was available per phase were included in the analyses. We used a total of
224 10 cycles for the face (2 cycles for 2 females, 1 cycle for 6 females) and 8 for the hindquarters (1
225 cycle per female) for the analysis. A median of 12 images were available per 28-day ovarian
226 cycle for facial skin (range: 10-15) and 11 for hindquarter skin (range: 7-14).

227

228 We performed general linear mixed models (GLMM) to examine whether R/G varies in such a
229 way as to reveal information about the timing of the fertile phase. GLMM is an extension of the
230 general linear model that accounts for repeated measurements of the same subject and for
231 unbalanced sample size by including random factors in the model. We analyzed the effect of a
232 continuous fixed variable, “day to estimated fertile phase” on R/G values and included “female
233 identity” and “cycle number” as nested random factors. In this analysis, the 5 days of the fertile
234 phase were all numbered 0; the day directly preceding the fertile phase was labeled day -1, the
235 day directly following it labeled day 1, and so on (Higham et al. 2008; 2009). First, we tested
236 whether R/G throughout the 28-day ovarian cycle follows a quadratic curve (i.e. highest values
237 reached at mid-cycle when the fertile phase occurs). Because GLMMs test for linear
238 relationships, we squared the numbers of the scale “day to estimated fertile phase” (Higham et
239 al. 2008; 2009). Next, in order to verify whether R/G values were higher during the fertile phase
240 compared to each of the other 2 phases, we tested the effect of the “day to estimated fertile
241 phase” on R/G values during the 10-day periods spanning 2 of the 3 defined phases (fertile vs.
242 pre-fertile, and fertile vs. post-fertile). Given potential problems associated with GLMMs and
243 small samples sizes as ours, we also carried out non-parametric statistics (Friedman tests with

244 post-hoc Wilcoxon signed-rank tests) using one cycle per female (the cycles for which R/G data
245 was available for both facial and hindquarter skin) to confirm our results. The two types of
246 analysis gave results with similar levels of statistical significance and we therefore present only
247 the results of the GLMMs here. Statistical analyses were undertaken in SPSS 17.0. All statistical
248 analysis were two-tailed and significance levels set at $p < 0.05$.

249

250 RESULTS

251

252 R/G of both the face and the hindquarters varied significantly throughout the ovarian cycle
253 when the entire 28 days of the cycle were considered; R/G values rose as the probability of
254 fertility increased and fell as the probability of fertility decreased (facial skin: $F=13.914$,
255 $p < 0.001$; hindquarter skin: $F=5.977$, $p=0.017$; Figure 3). Facial R/G was significantly higher
256 during the fertile phase compared to the 2 other phases (fertile vs. pre-fertile: $F=23.257$,
257 $p < 0.001$; fertile vs. post-fertile: $F=8.958$, $p=0.005$), but no such pattern was found for
258 hindquarter R/G (fertile vs. pre-fertile: $F=2.932$ $p=0.097$; fertile vs. post-fertile: $F=4.102$,
259 $p=0.053$)(Figure 4).

260

261 DISCUSSION

262

263 Using an objective and quantitative measure of color, along with estimates of ovulation date
264 based on measurements of fecal progesterone and estrogen metabolite levels, we have shown
265 that red skin coloration (R/G) for two regions of free-ranging female rhesus macaque sexual

266 skin significantly varies throughout the ovarian cycle in such a way that R/G values increase as
267 the probability of fertility rises. Facial R/G values were significantly higher during the fertile
268 phase compared to the 5-day periods immediately preceding and following it, but such a
269 pattern was not found for hindquarter R/G. Therefore, although sexual skin color appears to
270 contain general information about the probability of fertility during the ovarian cycle in rhesus
271 macaques, only facial skin color seems to contain more reliable information about its timing.
272 Skin color in this species, which lacks a prominent swelling, therefore appears to contain similar
273 information about the timing of the fertile phase as has been shown for swelling size in other
274 catarrhine primates (graded-signal hypothesis: Nunn 1999; e.g., Deschner et al. 2004; Brauch et
275 al. 2007; Higham et al. 2008).

276
277 Previous descriptive studies using subjective color measures have also reported that
278 hindquarter color varies throughout the ovarian cycle and is most intense during mid-cycle (the
279 presumed time of the fertile phase) in single-caged rhesus macaque females (Baulu 1976; Czaja
280 et al. 1977). Our results suggest that the period during which the highest R/G values are
281 reached includes, but might exceed, the fertile phase and thus only contains partial information
282 regarding its timing. In the only other study to combine detailed hormonal data with an
283 objective measure of hindquarter sexual skin color, Higham et al. (2008) showed that, in olive
284 baboons, the color of the sexual swelling does not contain information regarding the timing of
285 the fertile phase. Although more studies are needed, it appears that in cercopithecines, color
286 changes in the skin of the anogenital region and its surrounding areas contain some information
287 regarding the probability of fertility, but perhaps only in the absence of sexual swellings.

288

289 In addition to color in the hindquarters, Baulu (1976) also examined facial coloration in rhesus
290 macaques and found that it did not show cycle-related changes. In contrast, our results suggest
291 that facial color change contains reliable information about the timing of the fertile phase in
292 this species. The discrepancy between these studies may be attributed to the accuracy of
293 measurements. Baulu (1976) measured color based on weekly observer ratings and estimated
294 the timing of ovulation from menstruation date, both of which might not produce reliable data.
295 Our results for facial coloration are in accord with studies in other primate species: facial skin
296 was reddest during the periovulatory period of Japanese macaques (Fujita et al. 2004) and
297 mandrills (*Mandrillus sphinx*; Setchell et al. 2006), although it should be noted that these
298 studies used either an objective measure of color (Setchell et al. 2006) or reproductive status
299 (Fujita et al. 2004), but not a combination of the two. More studies using objective measures to
300 investigate the role of color change as a sexual signal in areas outside the anogenital region are
301 clearly needed.

302

303 In order to establish whether skin color change in rhesus macaques acts as a signal of the timing
304 of the fertile phase, it is crucial to determine whether males can perceive and interpret the
305 information contained therein (Snowdon 2004; Maynard-Smith and Harper 2005). An effective
306 way to achieve this is with an experimental approach that examines the impact of skin color
307 variation on male behavior in isolation from other potential signals and cues of female
308 reproductive status (e.g. female behaviors; Engelhardt et al. 2005). In pioneering experiments,
309 Waitt et al. (2006) showed that single-caged rhesus macaque males gaze longer at red than

310 non-red images of female hindquarters (but showed no difference for female faces), while
311 Deaner et al. (2005) found no effect of skin redness in the motivation of rhesus macaque males
312 (as measured by juice sacrifice) to view female faces or hindquarters. As detailed reproductive
313 hormone data were unavailable in these two experiments, it is unknown exactly what stage of
314 the ovarian cycle the images used represented, which could have influenced results. Male
315 rhesus macaques do pay selective attention to red color associated with pregnancy in images of
316 female faces (Gerald et al. 2009), thus red facial coloration is able to attract male attention.
317 However, skin color in the faces and hindquarters of females may contain information other
318 than the timing of the fertile phase which may or may not be of interest to males, such as age
319 (Strum and Western 1982), degree of sociability (Waitt et al. 2006) or parity (Gauthier 1999;
320 Setchell et al. 2006; Higham et al. 2008). In order to investigate skin color change as a signal of
321 the timing of the fertile phase in this species further, more experiments are required using
322 stimuli based on detailed reproductive hormone data. Future experiments should also ideally
323 be designed in a manner that takes into account the specifics of the rhesus visual system
324 (Stevens et al. this issue).

325

326 If skin color does act as a visual signal of the timing of the fertile phase, it remains unclear why
327 rhesus macaques have secondarily lost sexual swellings in their evolution only to express the
328 information about the timing of the fertile phase with a different signal. Perhaps the costs of
329 color change are less than those associated with swellings (e.g. increased body weight, parasite
330 loads, risks of injuries, and water retention; reviewed in Nunn 1999). As skin color change in the
331 perineal area may be less conspicuous than swelling size, it may be more visible if it covers a

332 larger skin surface: legs, thighs, tails and face. The sexual skin on the chest and abdomen of
333 gelada baboons has been explained by the large amount of time this species spends sitting on
334 the ground feeding, which hides the anogenital area (Dixson 1983; 1998). This explanation
335 could also apply to rhesus macaques since they may be one of the most terrestrial macaque
336 species (Napier and Napier 1967): wild rhesus macaques spend a significant amount of time
337 feeding on herbs and grass in some populations (Goldstein and Richard 1989), there is variation
338 in diet and habitat use between sites (e.g., Lindburg 1977; Seth et al. 2001). Although we know
339 little about the ecological conditions under which rhesus macaques evolved, it is likely that an
340 ecological force would be at play in the evolution of a sexual signal in the skin in the upper
341 body. If color change is more visible to potential male receivers in the face than in the
342 hindquarters in rhesus macaques, it may be that facial skin color change is more likely to have
343 been selected as a reliable signal of the timing of the fertile phase, as is suggested by our
344 results.

345

346 It is important to note that changes in color may occur more quickly and less predictably than
347 changes to the size of a swelling since stress, emotion and social interactions may affect blood
348 flow, and thus skin redness, in a short-term manner independent of reproductive hormones
349 (Changizi et al. 2006; Bradley and Mundy 2008). If the information contained in color change
350 can be interpreted by males, this information could perhaps be used more effectively by those
351 males who can monitor females on a regular basis (e.g., during a long consortship: Higham et al.
352 2009). Moreover, as baseline and maximal colors vary between females in this species (Brent et
353 al., unpublished data), previous experience with a given female may be crucial to the

354 interpretation of the signal. Information regarding the timing of the fertile phase may therefore
355 be unevenly distributed among males, which may potentially allow females to alter costs and
356 benefits of male monopolization and bias paternity toward preferred males (Nunn 1999; van
357 Schaik et al. 1999). A combination of behavioral and genetic data, along with objective
358 measurements of hormones and color, may shed light on the function of sexual skin color in
359 rhesus macaques, and lead to a greater general understanding of the evolution of sexual
360 signaling in primates.

361

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517

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519

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540 Figure 1: Location of sexual skin in female rhesus macaques. Squares and dashed lines illustrate
541 how the areas in which color was measured were selected (enlarged versions of 10x10 pixels,
542 see Methods for details).

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545 Figure 2: Composite hormonal profile of the 10 ovarian cycles included in this study. Black line:
546 PdG; dashed gray line: E1C. Values represent means + SEM.

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548 Figure 3: Composite sexual skin color profile throughout the ovarian cycle. Values represent the
549 mean percentage of maximum R/G reached for each cycle. N=10 cycles for facial skin, N=8
550 cycles for hindquarter skin.

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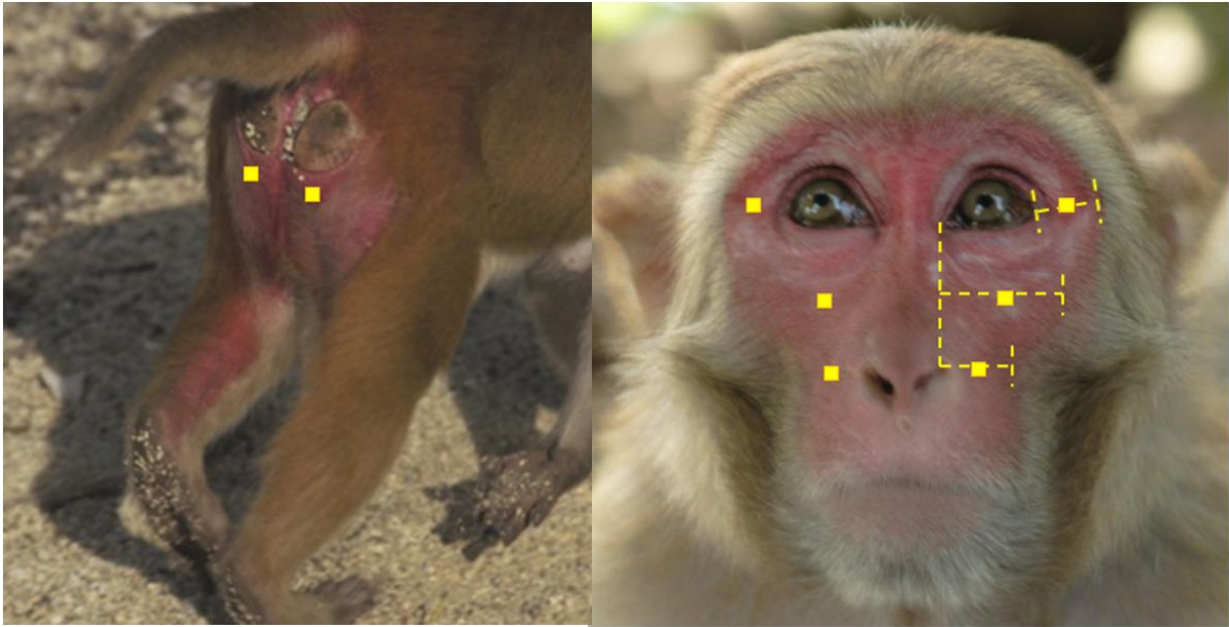
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553 Figure 4: Red to green ratio for the cycles of individual females for the 3 defined phases. N=10
554 cycles for facial skin, N=8 cycles for hindquarter skin.

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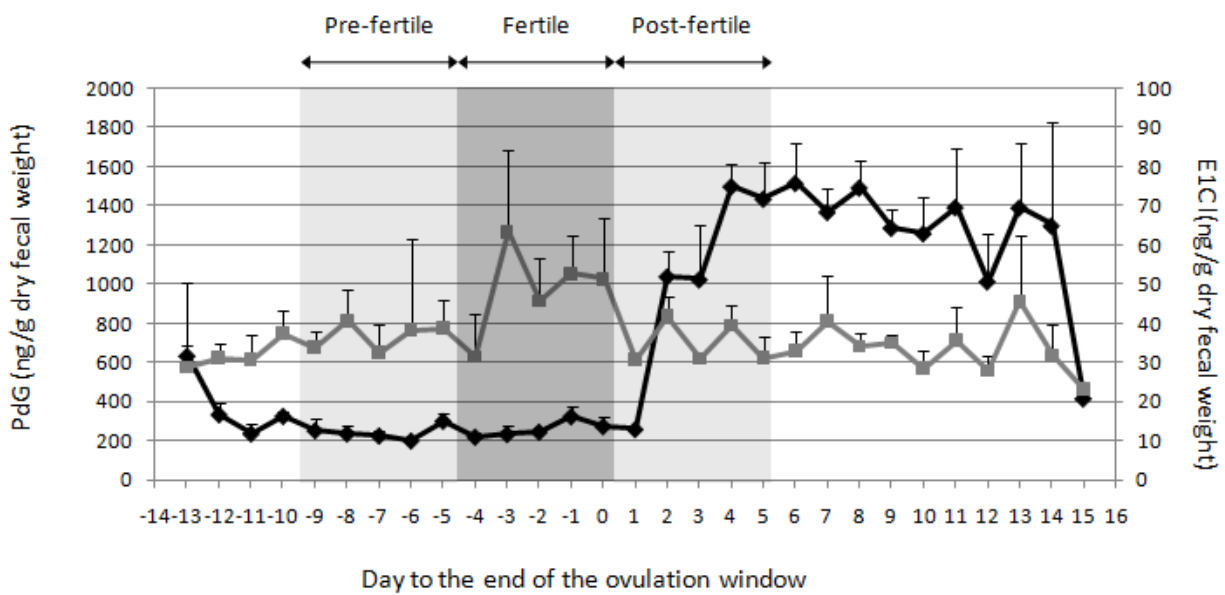
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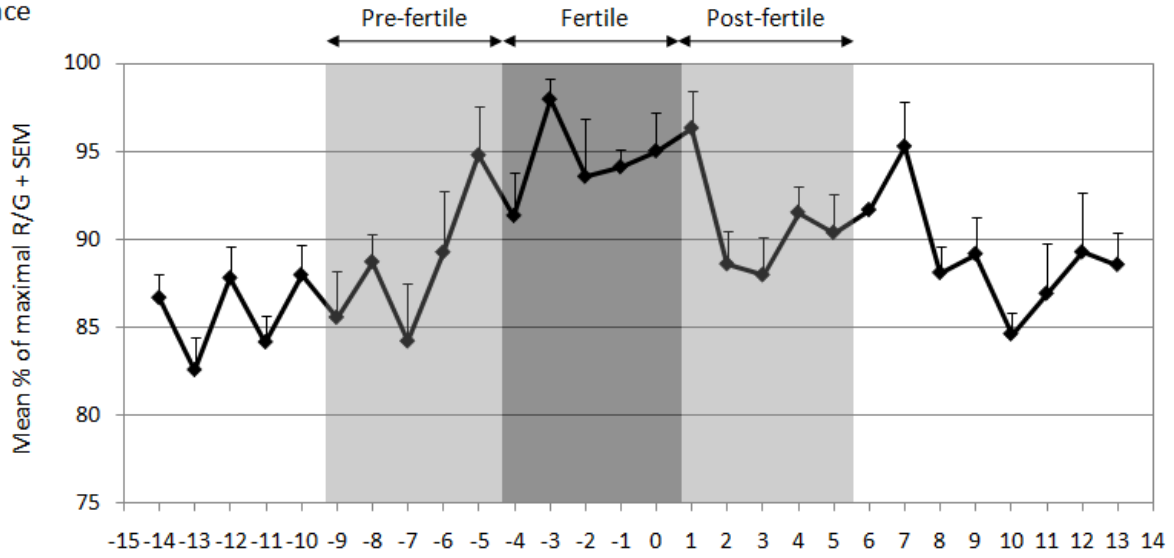


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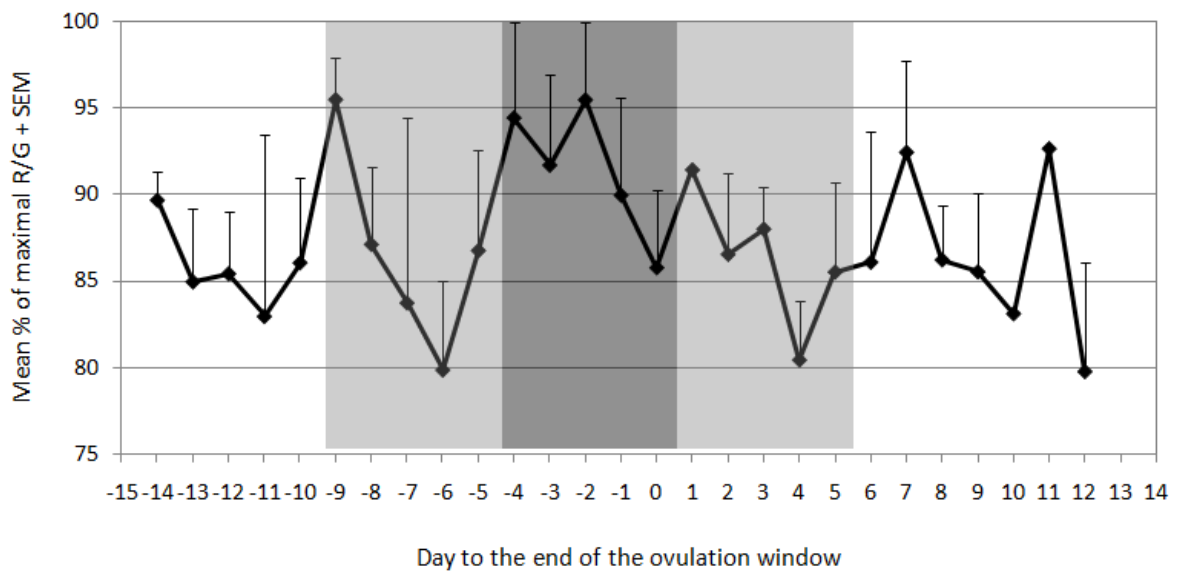
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Face



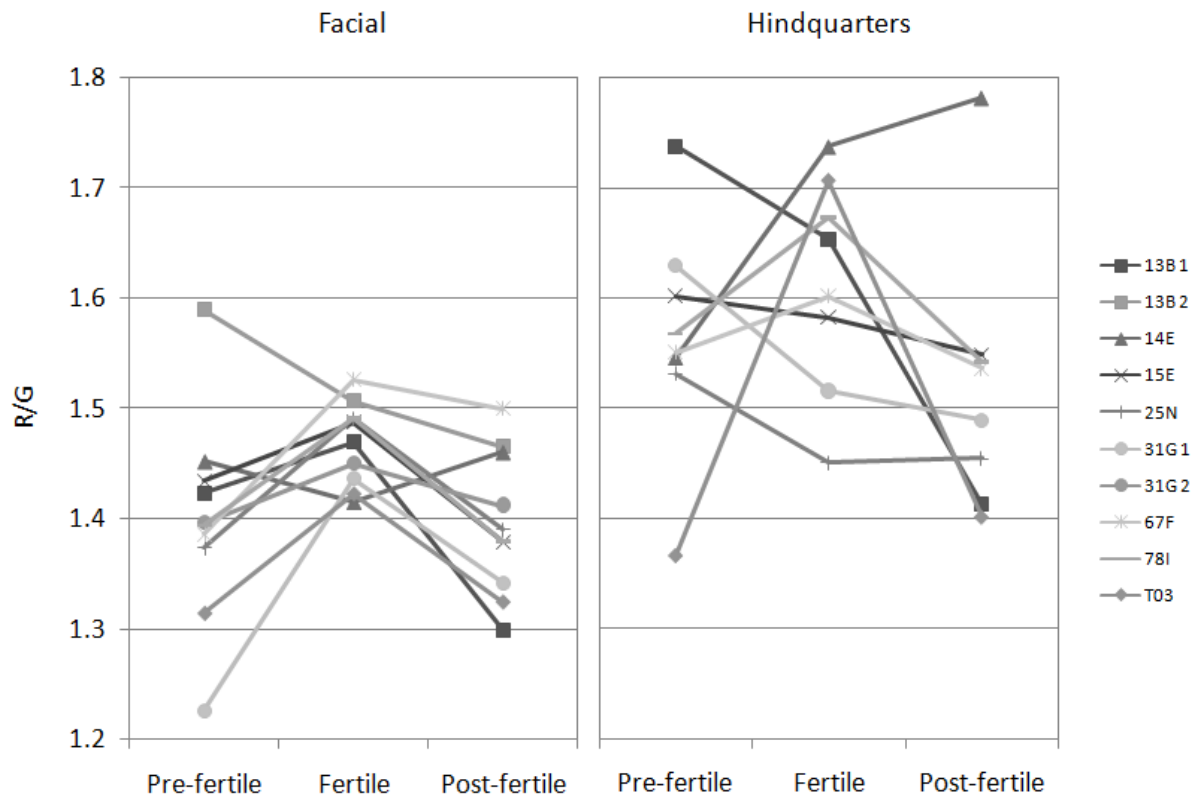
Hindquarters



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Dear Dr. Higham,

Many thanks for the reviews of our manuscript (#IJOP-D-09-00025) and the invitation to resubmit this paper. We were pleased to note that you and the two referees found that our study was an interesting contribution to the literature on color signaling in primates. We have now revised the paper in line with the comments received. We detail below the way in which we have dealt with the points raised by each of the referees in turn.

We thank you for your continued consideration of this manuscript.

Yours sincerely,

Constance Dubuc, Lauren Brent, Amanda Accamando, Melissa Gerald, Ann MacLarnon, Stuart Semple, Michael Heistermann and Antje Engelhardt

Comments from the Editor (Primate Coloration_Color Vision)

Both Reviewers and I are in agreement that your manuscript represents an interesting contribution to the literature on color signaling in primates, being one of the few studies so far to have combined quantitative and objective measures of both coloration and the female endocrine cycle. However, some revisions are necessary before the manuscript might be acceptable for publication in IJP. I would like to highlight a few of the Reviewer comments, and add some of my own.

1) I agree with Reviewer 2 that more information about the error contained in your method would be helpful, especially with regards to how consistent your R:G measurements are (especially for example, for each female on a given day). If there is variability in this, is this because of error in the method, or is it because, as Reviewer 1 queries, there is variation within a female within a day?

There is only one good image available for the same female on a given day and thus we cannot conduct the analysis suggested here. As we expect color to vary day-to-day throughout the reproductive cycle, calculating intra-individual color variation using pictures taken during different days does not appear to be a good alternative. However, we now provide more details about the method to respond to the other issues raised by Reviewers 1 and 2 (see P6 L131-136; P 8 L162-171).

2) Both Reviewers would have liked some analysis of the multiple cycles available for each female. However, I only partly agree with Reviewer 2 that it would be better to undertake mixed-modeling on these data to enable you to analyze the whole dataset at the same time. This is because, although you have discarded data at present (which is not ideal), using GLMMs on very small datasets is not ideal either. Have you tried undertaking the analyses both ways - firstly, as you do it now with only one cycle per female, and secondly using GLMMs on the whole dataset with multiple cycles from the same female accounted for by the inclusion of random factors in the model? If the two sets of analyses produced results that did not differ in

their significance, then this would be very convincing, and you would only have to present one set, stating in the methods that it makes no difference how the data are treated. This would head off any concerns from readers who might, like Reviewer 2, feel that mixed models are the way forward for these data.

We thank both the editor and reviewer 2 for their comments on the statistical method used. Analyses conducted with GLMMs and non-parametric tests did not differ in their significance, which is stated in the methods, as suggested. We present the results of the GLMMs only because it allows us to conduct regression, which gives more refined information concerning whether color change contains information about the timing of the fertile phase. Please see changes in the Methods (P6 L121-124, P10 L210-213, P11 L223-226 and 228-248, Results (P12 L252-259), and Discussion (P12 L265-2741, P13 L2798-286 P16 L340-343) sections.

3) Looking at Figure 3, it seems to me that, while hindquarter color does not indicate the timing of the fertile phase, it does seem to rise significantly above all other values around the time of ovulation specifically. I am surprised that you have not attempted to analyze this. Although we can usefully define an assumed fertile period around the estimated ovulation date, in which we treat each day as being of equal conceptive probability, the authors are surely aware that this is not really the case, and that there is really a function of conceptive probability within this fertile phase that is likely to be much higher around ovulation than, say, 3 days before it (when the female may be fertile, but with low probability of conception). From Figure 3, it looks like the clearest result of all was a sudden rise in hindquarter R:G around ovulation, which would match conceptive probability quite well probably. Of course it might not be possible to detect this statistically if you focus only on R:G through the fertile phase generally, as for most of this period the R:G of the hindquarters does not differ from any other part of the cycle. Is it possible to undertake an analysis of color change specifically with respect to ovulation timing versus other parts of the cycle, rather than the fertile phase more generally?

We thank the editor for raising this point. However, giving the gap in sample collection before the significant PdG rise, we consider that the estimated day of ovulation cannot be established precisely enough to conduct analyses as suggested by the editor. Moreover, the figure 3 generated with the new data set no longer supports the potential pattern described here.

Some minor editorial comments are:

1) In the abstract I think it is better to stick to the present tense (i.e. 'we show' rather than 'we showed' (lines 33 & 38).

These changes have been made.

2) There is more to fertility than just intra-cycle likelihood of conception - the term also includes inter-cycle differences in the likelihood of conception. As you do not attempt to assess

whether there were differences in color between conceptive and non-conceptive cycles (which you could have looked at in your dataset), I would make it clear that your analysis is of color changes associated with the intra-cycle likelihood of conception. This concerns the Abstract (line 25), where you state that you investigate "whether changes in sexual skin color contain information about fertility". Please clarify this. Also in the Introduction (pg 3, line 38), you state that "Given that changes in the sexual skin of cercopithecines occur over the ovarian cycle, they may contain information regarding when females are most likely to be fertile", whereas I think you mean that they may contain information regarding the intra-cycle likelihood of conception specifically.

We thank the editor for this input. We now use the expression "the timing of the fertile phase" or /within cycle probability of fertility" instead of "fertility" (Please see P2 L27-28, P3 L63-64, P4 L78, P5 L91-92, P14 L290, P15 L302-303).

3) Pg 4, line 53 - Is 'raspberry red' a defined color? If not is it the best description? It seems to me to be a culture-specific color reference that may be lost on many primate habitat country readers.

We now use the expression "deep red" which is more neutral (P4 L88).

4) It would be helpful to add continuous line numbers to the manuscript for the revision.

We added new numbering. We hope that the presence of two sets of line numbering will not be confusion during the review process (as the IJP automated system adds non-continuous line numbering when the manuscript is transformed in .PDF format).

Reviewer #1:

This paper examines the possible association between skin color changes and ovulation in rhesus macaques. The manuscript is logically framed and well-written, and the results (namely that facial skin color might indicate fertility but rump skin color probably does not) provide an important and interesting complement to previous work examining how male rhesus macaques attend to female face and rump coloration. This is one of the few studies with matched hormonal data indicating fertility and objective measures of skin coloration. And, although it is a relatively small sample, it represents an important first step in understanding the evolution and maintenance of this striking aspect of primate coloration.

The manuscript is well worth publishing in IJP and requires only minor modification.

In fact, my only major criticism is that more information should be given on the timing and context in which the photographs were taken. Were photos always taken at the same time of day and within the same behavioral context (e.g. feeding)? How much does individual skin color vary throughout the day?

Thank you for bringing this to our attention. We now provide this information in the methods section (See Methods P6 L133-136). As the images were captured around the same time every day, during a period of travelling and feeding, there should not be biases in our data due to the time of day or behavioral context.

The methods state that only one ovarian cycle was used for each female in order to avoid repeated measures of the same individuals, but were females tested over >1 cycle? If so, was there individual variation, or were results consistent, across cycles? It would be helpful to clarify these things in the methods.

The change in statistical method (GLMM) allows us to use all cycles for which we have enough hormonal and color data in order to establish a pattern. However, there are only two females for which two cycles are available and thus there are not enough data to conduct intra-female comparisons. Figure 4 now illustrate both inter- and intra-individual variation.

Reviewer #2:

This study focuses on primate skin coloration in the context of sexual signaling, investigating whether the variations of skin color in two locations of the female body (the face and the hindquarters) reflect the timing of the fertile phase of the menstrual cycle in rhesus macaques, a species which does not produce sexual swellings. Their results show that facial color is significantly redder during the fertile phase than during the pre- and post- fertile phases, whereas the colorations of hindquarters do not show such variation. These results are discussed in the context of previous results and within an evolutionary perspective.

The manuscript is overall clearly written, relies on well-described, apparently reliable methodology and the results answer the research questions. This study does not tackle a new question, as several studies have already investigated the links between sexual skin or facial color variations and the different phases of female menstrual cycle. However, this study tackles these issues using more reliable methods, i.e. quantitative assessment of skin coloration and hormonal measures indicating the timing of ovulation.

Some minor comments are listed below using page and line numbers:

P2, L. 28. Maybe change "Females do express changes in the..." into "Females do express visually detectable changes in the..."?

The change has been made (P2 L33).

P2. L. 33 to 35. The study does not present any test showing that the coloration of the hindquarters varies throughout the cycle. And the extent of variation present in Figure 3 is too important to make such conclusion solely from the visual inspection of the graph. It is also not mentioned in this graph whether the error bars display confidence intervals (CI) and if so, the

alpha value to draw these CI, or the range of values observed in the sample. This does not help to make inferences directly from the graph without statistical test. This comment also concerns the presentation of the results P. 11, lines 51-59.

We thank reviewer 2 for this comment. We now present statistical analysis showing that coloration significantly varies throughout the cycle in the hindquarters.

As for the error bars, we used S.E.M. and the information is available in the figures and their title.

The introduction and discussion present repeats that could be avoided. For instance, the idea that studies using objective assessments of color and reproductive status are required appears at least 3 times:

- P4, L. 35-43
- P5, L. 28-36.
- P13, L. 20-21.

P5, L.49-54. "This constitutes the first study..." I leave this to the authors but this kind of sentence is often discouraged. Authors have already clearly presented the novelty of their study compared to previous ones within the 2 previous paragraphs, so this sentence does not add much.

We appreciate the comments from Reviewer 2 regarding these matters of style. After consideration, we decided not to change our phrasing as we feel it helps readers understand in what context the study was conducted, especially as the article will be published in a special issue on color studies in primatology.

P5, L.48: It is perfectly clear that this study examines whether color changes reveal information about the timing of fertile phase but it is not clear to me how this study examines to what extent these changes reveal information about the timing of fertile phase. There is nothing mentioned in the results regarding this, and the statistics do not compare quantitative variables (which could, for instance, generate quantitative estimates describing the relationship linking color changes and hormonal levels) but assess the effect of a categorical factor (menstrual cycle phase) on a quantitative variable (R/G ratio in sexual skin). So I would suggest deleting this third aim, or finding a way to justify it at some point.

Thank you for bringing this to our attention. We rephrased the aims in the introduction in a way that fits this comment (P5, L107-108 verify lines). We think that this new phrasing should satisfy Reviewer 2's concern, and that it is actually a more appropriate way to express the aims of this study. Moreover, it should be noted that we now assess the impact of a quantitative variable on R/G values instead of assessing the effect of a categorical factor.

P6, L. 30-34 "To avoid a disproportionate contribution of individual females to the dataset, we used one ovarian cycle per female..*". There are statistical methods that allow taking into

account repeated measures per individual in an observational dataset (e.g. mixed-model procedures). The statistical approach used is correct, but does not exploit the full potential of the dataset. A great deal of efforts has been provided to use powerful methods (by combining endocrinological data with objective measures of color) in generating the data, but the statistical approach constrains to discard part of the dataset, and loses considerable power in analysing the remaining part... Which weakens the paper unnecessarily.

We thank Reviewer 2 for suggesting the use of this method. The change has been made (see our response to the editor for more details).

P8, L. 20. There is no explanation of how the locations of the pixel areas were chosen, or why these particular areas were selected. Figure 1 shows where these areas are, but does not provide any explanation of how the dashed lines were used to locate an area. That would be interesting because I suspect these areas may be difficult to locate depending on the angle of your photo. And with respect to this latter point, the methods do not provide any precisions regarding the angle of the shots, and whether any precautions were taken to minimize this possible bias.

More information regarding the selection of the pixel areas and the precautions taken to minimize bias is now provided in the method section (P6 L131-132, P8 L162-171).

There is no assessment of the error measure of the R/G ratio in the pictures: what is the mean variation of the R/G ratio between pictures of the same subject in the same location (i.e. face or hindquarters)? Such measure is essential to interpret the results of this type of methodological design, especially for the hindquarters, which seem to show a great deal of inter-individual variations. And providing evidence for a relatively low error measure would be a convincing argument to conclude that "your method measures color accurately" (P.8, L.7) (probably more than the one used here).

As mentioned in our comments to the editor, we do not have the data necessary to verify the measure error of our method as we did not collect more than one image per day per female for each region.

P14, l. 27-33. Some sexual swellings appear to be much more complex in terms of their signalling content than just expressing information about the timing of the fertile periods, e.g. the gradual signal hypothesis (Nunn 1999). It is unclear whether the color changes of the macaque faces are gradual, and more generally if the signal content of (some) sexual swellings and macaque facial skin are close (except for the signaling of the fertile period). So it is difficult (and it may well even be wrong) to state "If facial skin color does act as a visual signal of the timing of the fertile phase, it remains unclear why rhesus macaques have secondarily lost sexual swellings in their evolution, only to express the information it usually contains with a different signal".

We understand the reviewer's concerns. We rephrased this in a way that states more clearly that we are discussing only one of the possible signals conveyed by swellings (Please see P15 L326-327).

P14, L. 48. Should read "They may be one of the most..." or "They may be among the most..." but not "They may be among one of the most..."?

The change has been made (P15 L342).