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

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ABSTRACT

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

KEYWORDS

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

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).

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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*: Gesquiere et al. 2007; *Papio anubis*: Higham et al.

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

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

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

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

METHODS

Study site and subjects

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

Assessment of skin coloration

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

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

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

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

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

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

Assessment of the ovarian cycle and definition of the fertile phase

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

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

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

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

Data analysis and statistics

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

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

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

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

RESULTS

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

DISCUSSION

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

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

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

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

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

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

If 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 about the timing of the fertile phase with a different signal. Perhaps the costs of color change are less than those associated with swellings (e.g. increased body weight, parasite loads, risks of injuries, and water retention; reviewed in Nunn 1999). As skin color change in the perineal area may be less conspicuous than swelling size, it may be more visible if it covers a

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

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

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

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519

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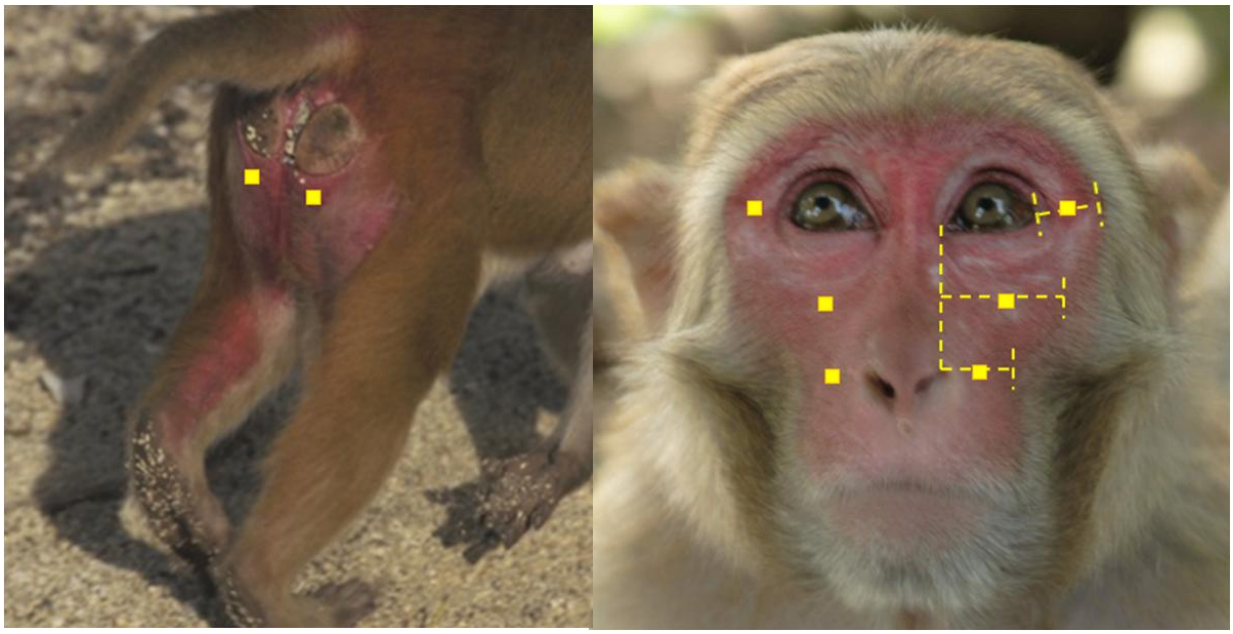
Figure 1: Location of sexual skin in female rhesus macaques. Squares and dashed lines illustrate how the areas in which color was measured were selected (enlarged versions of 10x10 pixels, see Methods for details).

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

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

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



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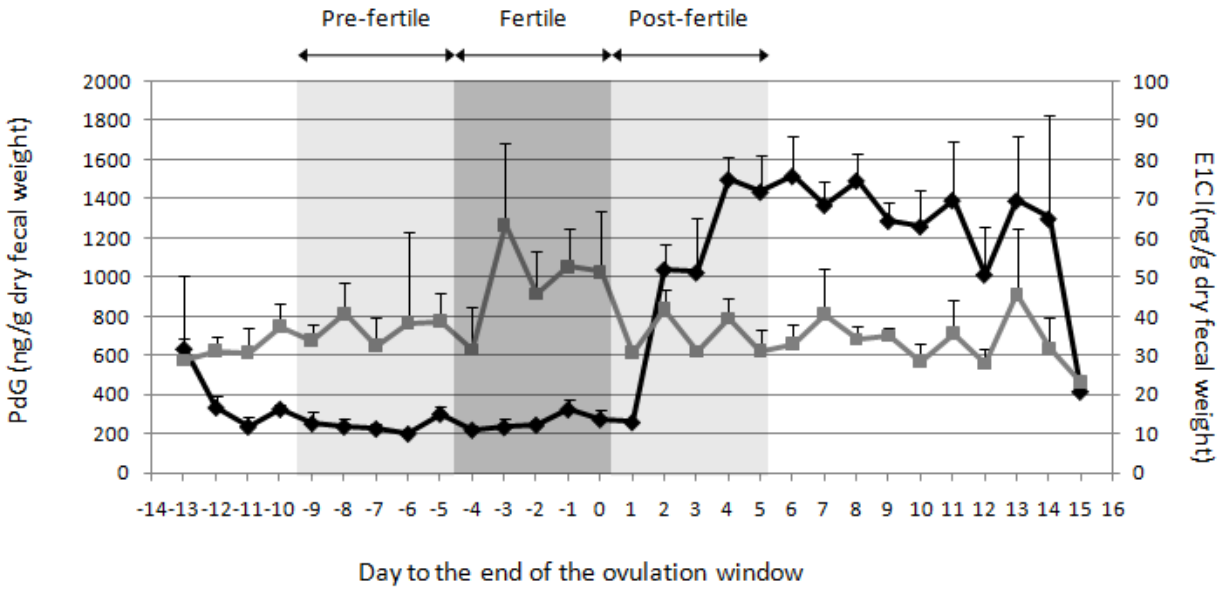
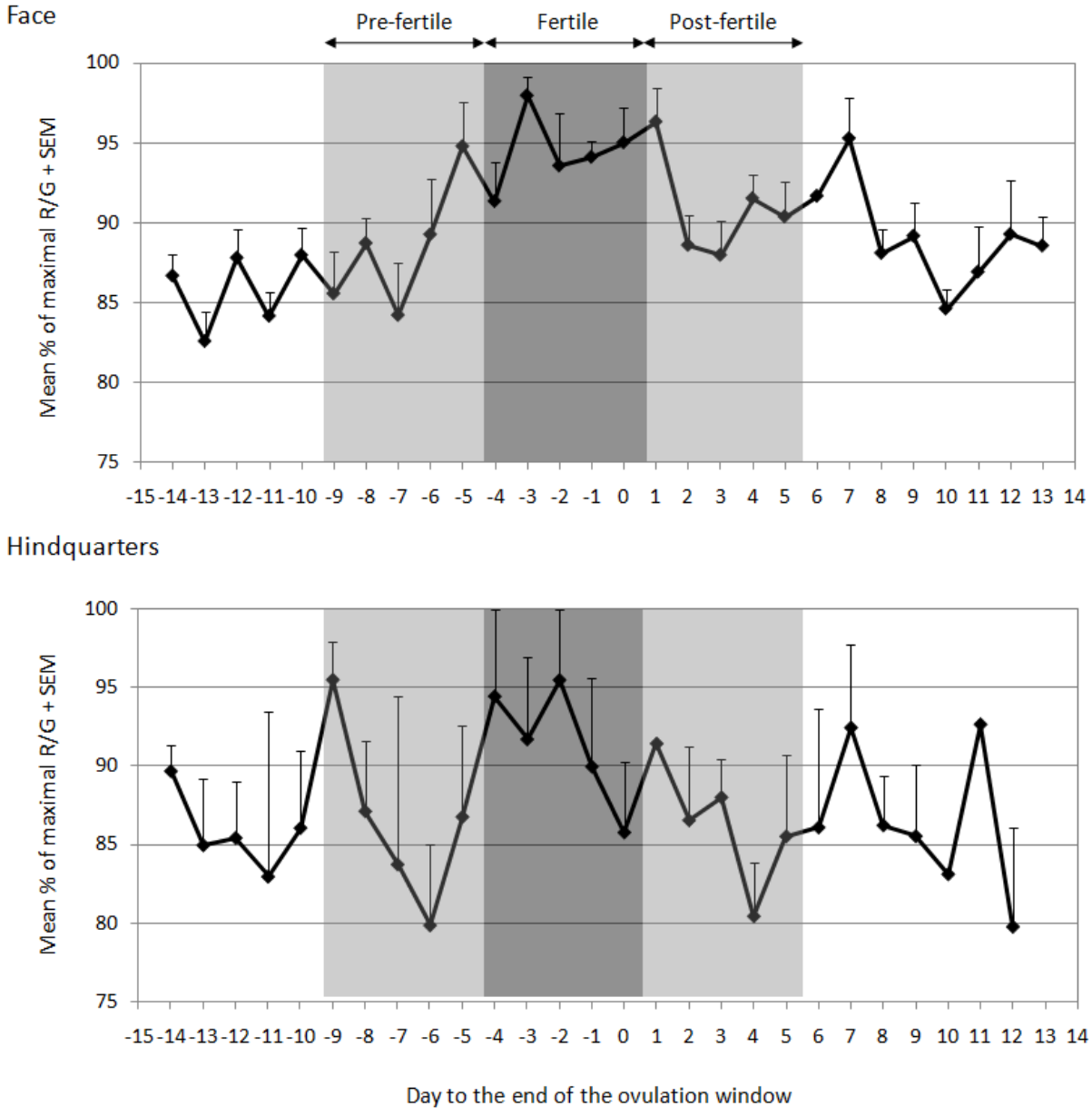

Pre-fertile
Fertile
Post-fertile
PdG (ng/g dry fecal weight)
E1C1(ng/g dry fecal weight)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
100
90
80
70
60
50
40
30
20
10
0
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Day to the end of the ovulation window

564

565

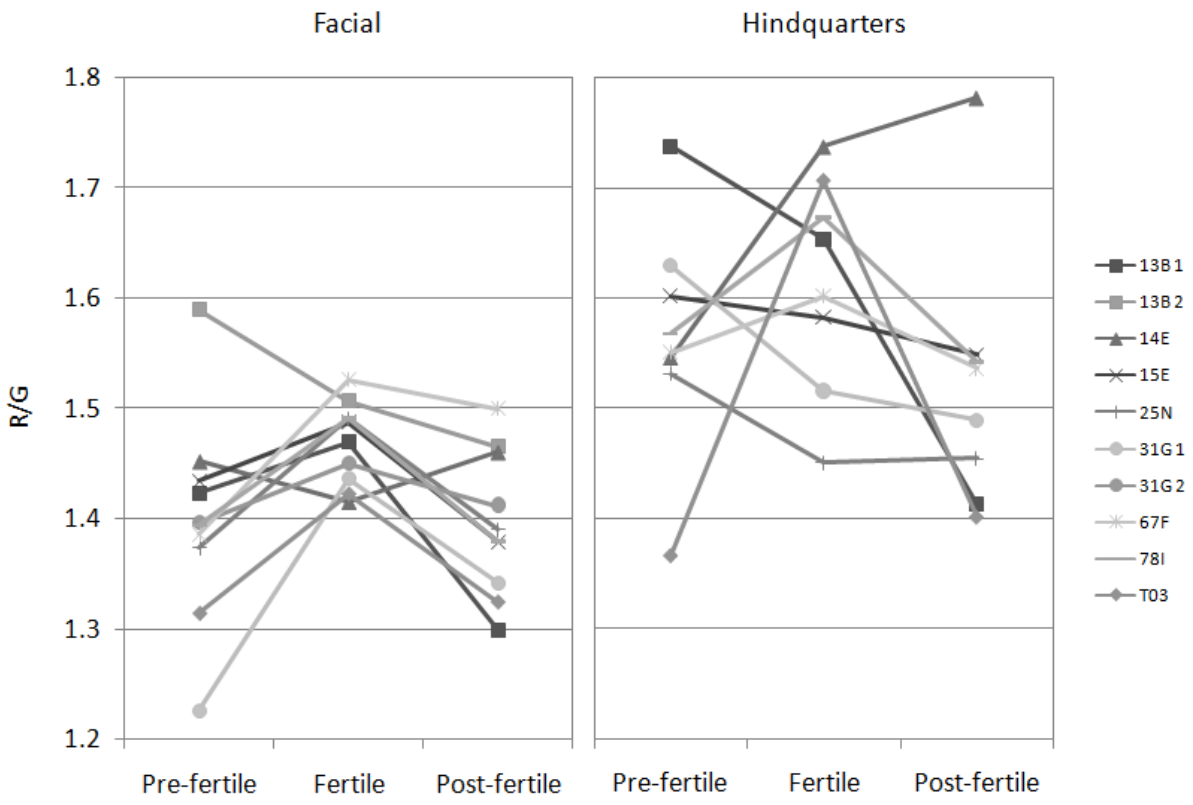
566


Face
Pre-fertile
Fertile
Post-fertile
Mean % of maximal R/G + SEM
100
95
90
85
80
75
-15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Hindquarters
Mean % of maximal R/G + SEM
100
95
90
85
80
75
-15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day to the end of the ovulation window

567

568

569


Facial
Hindquarters
R/G
1.8
1.7
1.6
1.5
1.4
1.3
1.2
Pre-fertile
Fertile
Post-fertile
Pre-fertile
Fertile
Post-fertile
13B 1
13B 2
14E
15E
25N
31G 1
31G 2
67F
78I
T03

570

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 228248, 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 hinquarters 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).