Seeing spots: Measuring, quantifying heritability, and assessing fitness consequences of coat pattern traits in a wild population of giraffes (*Giraffa camelopardalis*)

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RH: Giraffe coat pattern traits

ABSTRACT

Polymorphic phenotypes of mammalian coat color have been important to the study of genetics and evolution, but little is known about the heritability and fitness consequences of variation in complex coat pattern traits in wild populations. Understanding the current evolution of coat patterns requires reliably measuring traits, quantifying heritability of the traits, and identifying the fitness consequences of specific phenotypes. Giraffe coat markings are highly variable and it has been hypothesized that variation in coat patterns most likely affects fitness by camouflaging neonates against predators. We quantified spot pattern traits of wild Masai giraffes using image analysis software, determined whether spot pattern traits were heritable, and assessed whether variation in heritable spot pattern traits was related to fitness as measured by juvenile survival. The methods we described comprise a framework for objective quantification of mammalian coat pattern traits based on photographic coat pattern data. We demonstrated that characteristics of giraffe coat spot shape are heritable. We

did not find evidence for juvenile survival consequences of variation in spot traits, suggesting that spot traits are currently not under strong directional, disruptive, or stabilizing selection for neonate camouflage in our study population, but our sample size could not detect small differences in survival. Spot trait variation also may be more relevant to other components of fitness, such as adult survival or fecundity. We hope this case study will inspire further investigations of coat pattern traits.

Key words: adaptation, coat pattern, heritability, natural selection, phenomics, phenotypic selection, photogrammetry, quantitative genetics, remote measurement, visual animal biometry

INTRODUCTION

Complex coat patterns are found on many mammal species and these phenotypic traits are hypothesized to play adaptive roles in predator and parasite evasion, thermoregulation, and communication (Cott 1940; Caro 2005). Pigmentation biology has played a prominent role in the foundation of genetics and evolutionary biology with most work on vertebrates focused on a few starkly different color morphs in mice (Hoekstra 2006). However, most studies to date of the heritability and adaptation of complex animal skin pigmentation patterns have concentrated on fishes with their multiple chromatophores (Kelsh 2004). Researchers have proposed mathematical models for pattern-formation mechanisms in animal markings (Murray 1981; Maini 2004; Garvie and Trenchea 2014), and hypothesized the genetic and developmental mechanisms for the markings (Mills and Patterson 2009; Eizirik *et al.* 2010), and although studies of heritability and adaptation of coat patterns in wild populations are becoming more prevalent (Kruuk *et al.* 2008), we see a need for broadly applicable tools and techniques to quantify coat pattern traits in wild populations (Willisch et al. 2013).

Understanding the current evolution of a trait in a wild population requires reliably measuring individual variation in the trait, quantifying heritability as the proportion of observed phenotypic variation of a trait that is passed from parent to offspring, and assessing the fitness consequences of phenotypic variation (Lande and Arnold 1983; Falconer and Mackay 1996). The fraction of variability in a phenotypic trait that is explained by genetic factors is heritability, which can be estimated as the resemblance of the offspring to its parents (Falconer and Mackay 1996; Roff 1997). Fitness of a trait can be assessed in many ways, but because neonatal mortality in mammals is generally higher than any other age class (Lee and Strauss 2016), one common proxy of phenotypic fitness is juvenile survival (Paterson *et al.* 1998). Here we provide an example quantification and analysis of heritability

and fitness for mammalian coat pattern traits using giraffe (*Giraffa camelopardalis*) as the organism of interest.

Giraffe skin pigmentation is uniformly dark grey (Dimond and Montagna 1976), but the spots that make up their coat pattern markings are highly variable in traits such as color, roundness, and perimeter tortuousness, and this variation has been used to classify subspecies (Lydekker 1904), and to reliably identify individuals because patterns do not change with age (Foster 1966; Bolger *et al.* 2012; Dagg 2014). Dagg (1968) first presented evidence from a small zoo population that the shape, number, area, and color of spots in giraffe coat patterns may be heritable, but analysis of spot traits in wild giraffes, and tools for objectively measuring spot characteristics have been lacking. It has been hypothesized that giraffe coat patterns evolved to camouflage neonates whose primary defense against predation is concealment (Langman 1977; Mitchell and Skinner 2003); thus the most likely fitness effects from variation in coat patterns should be variation in juvenile survival. Alternative hypotheses about the adaptive value of giraffe coat markings include thermoregulation (Skinner and Smithers 1990), and in this social species with good visual sensory perception (Dagg 2014; VanderWaal *et al.* 2014), markings could also facilitate individual recognition (Tibbetts and Dale 2007) and kin recognition (Beecher 1982; Tang-Martinez 2001).

The spot patterns of Masai giraffes (*G. c. tippelskirchii*) are particularly diverse among giraffe populations (Dagg 1968; **Fig 1**). Masai giraffes spots vary in color and shape from those that are nearly round with very smooth edges (low tortuousness), to extremely incised or lobate (high tortuousness). Quantifying heritability and fitness consequences of phenotypic variation in coat pattern traits of giraffes could potentially contribute to the understanding of the evolution of mammalian coat patterns. Our purpose in this study was to 1) objectively and reliably quantify the spot pattern traits of wild Masai giraffes in northern Tanzania using photogrammetry and image analysis software, 2) determine whether spot

pattern traits were heritable from mother to offspring, and 3) determine whether variation in heritable spot pattern traits was related to fitness as measured by juvenile survival.

METHODS

Field Data Collection

This study used data from individually identified, wild, free-ranging Masai giraffes in a 1700 km^2 sampled area within a 4400 km^2 region of the Tarangire Ecosystem, northern Tanzania, East Africa. We collected data during systematic road transect sampling for photographic capture-mark-recapture (PCMR). We conducted 26 daytime surveys for giraffe PCMR data between January 2012 and February 2016. We sampled giraffes three times per year around 1 February, 1 June, and 1 October near the end of every precipitation season (short rains, long rains, and dry, respectively) by driving a network of fixed-route transects on single-lane dirt tracks in the study area. We surveyed according to Pollock's robust design sampling framework (Pollock 1982; Kendall *et al.* 1995), with three occasions per year. Each sampling occasion was composed of two sampling events during which we surveyed all transects in the study area with only a few days interval between events. Each sampling occasion was separated by a 4-month interval (4.3 years $\times$ 3 occasions $year^{-1}$ $\times$ 2 events $occasion^{-1}$ = 26 survey events).

During PCMR sampling events, a sample of individuals were encountered and either "sighted" or "resighted" by slowly approaching and photographing the animal's right side at a perpendicular angle (Canon 40D and Rebel T2i cameras with Canon Ultrasonic IS 100 – 400 mm lens, Canon U.S.A., Inc., One Canon Park, Melville, New York, 11747, USA). We identified individual giraffes using their unique and unchanging coat patterns (Foster 1966; Dagg 2014) with the aid of pattern-recognition software Wild-ID (Bolger *et al.* 2012). We attempted to photograph every giraffe encountered, and recorded sex and age class based on physical characteristics. We categorized giraffes into four age classes: neonate calf (0 – 3

months old), older calf (4 – 11 months old), subadult (1 – 3 years old for females, 1 – 6 years old for males), or adult (> 3 years for females, > 6 years for males) using a suite of physical characteristics (Strauss *et al.* 2015), and size measured with photogrammetry (Lee *et al.* 2016a).

All animal work was conducted according to relevant national and international guidelines. This research was carried out with permission from the Tanzania Commission for Science and Technology (COSTECH), Tanzania National Parks (TANAPA), the Tanzania Wildlife Research Institute (TAWIRI). No Institutional Animal Care and Use Committee (IACUC) approval was necessary because animal subjects were observed without disturbance or physical contact of any kind.

Quantification of Spot Patterns

We analysed spot traits of each animal within the shoulder and rib area by cropping all images to an analysis rectangle that fit horizontally between the anterior edge of the rear leg and the chest, and vertically between the back and where the skin folded beneath the posterior edge of the foreleg (**Fig 1**). We quantified spot characteristics of each animal's pattern using the Color Histogram and Analyze Particles procedures of ImageJ (Schneider *et al.* 2012). For color analysis, we used the entire analysis rectangle and full-color photos. For spot measurements we analysed 8-bit greyscale images that we converted to bicolor (black and white) using the ImageJ Enhance Contrast and Threshold commands. To account for differences in image resolution and animal size (including age-related growth), we set the measurement unit of each image equal to the number of pixels in the height of the analysis rectangle. Therefore all measurements are in giraffe units (GU), where 1 GU = height of the analysis rectangle (**Fig 1**). We excluded particles cut off by the edge of the analysis rectangle to avoid the influence of incomplete spots, and we also excluded spots whose area was <0.00001 GU^2 to eliminate the influence of speckles.

We characterized each animal's spot pattern traits within the analysis rectangle using the following 11 metrics available in ImageJ: number of spots; mean spot size (area); mean spot perimeter; mean angle between the primary axis of an ellipse fit over the spot and the x-axis of the image; mean circularity ($4\pi \times [Area] / [Perimeter]^2$ with a value of 1.0 indicating a perfect circle and smaller values indicating an increasingly elongated shape); mean maximum caliper (the longest distance between any two points along the spot boundary, also known as Feret diameter); mean Feret angle (the angle [0 to 180 degrees] of the maximum caliper); mean aspect ratio (of the spot's fitted ellipse); mean roundness ($4 \times [Area]\pi \times [Major\ axis]^2$ or the inverse of *aspect ratio*); mean solidity ([*Area*] / [*Convex area*], also called tortuousness); and mode shade ([65536 × r] + [256 × g] + [b] using RGB values from color histogram from full color photos).

We quantified among-individual variation in trait values by reporting the mean, SD, and coefficient of variation (CV) of each trait. We quantified the repeatability of our spot pattern trait measurement technique by computing the amount of among-measurement variation made on different photos from different dates for the same animal using a set of 30 animals with >2 images per animal.

Heritability of Spot Traits

Parent-offspring (PO) regression is one of the traditional quantitative genetics tools used to measure heritability (h^2; Falconer and Mackay 1996). PO regression compares phenotypic trait values in parents to those same trait values in their offspring, with the slope of the linear regression line between the mean parent phenotype and the mean offspring phenotype providing an estimate of the heritability of the trait. Our analysis of maternal-offspring regression and the estimated slope represents one-half of total heritability. Advantages and disadvantages of the parent-offspring method to estimate heritability compared to other methods such as the half-sibling method or the animal model have been elucidated (Åkesson

et al. 2008; de Villemereuil 2013), with the primary advantage when studying wild populations being that PO regression requires less information about family structure and only one offspring per individual (Roff 1997; Lynch and Walsh 1998). While PO regression can have low statistical power and inherent biases when estimating heritability due to environmental effects shared by related individuals or unbalanced sampling (Wilson *et al.* 2010), this methodology was the most appropriate for our study design because we were unable to identify fathers, and our sample did not include any maternal siblings or half-siblings.

We identified mother-calf pairs by observing extended suckling behavior. Wild female giraffes very rarely suckle a calf that is not their own (Pratt and Anderson 1979). We examined all identification photographs for individuals in known mother-calf pairs, and selected the best-quality photograph for each animal based on focus, clarity, perpendicularity to the camera, and unobstructed view of the torso. We found 31 known mother-calf pairs with high-quality photographs of both animals.

We predicted spot pattern traits of a calf would be correlated with those of its mother but not with a random cow. For comparison of spot characteristics between known mother-calf pairs, we created a null expectation set of random cow-calf pairs using the same photographs by assigning a random mother to each calf (without replacement and without pairing a mother with her own calf). We tested our prediction for each spot characteristic using simple linear regressions of calf values versus mother values (heritability models), and calf values versus random cow values (null expectation models). Because we examined 11 spot traits, we used the Bonferroni adjustment (α/number of tests) to account for multiple tests and set our adjusted $\alpha = 0.0045$. We performed statistical operations using the lm function in R (R Core Development Team 2013).

Fitness of Spot Patterns from Juvenile Survival

We assembled encounter histories for all calves first observed as neonates for survival analysis. For each calf we selected the best-quality calf-age (age <6 mo) photograph based on focus, clarity, perpendicularity to the camera, and unobstructed view of the torso. We analysed survival using capture-mark-recapture apparent survival models. No mark-recapture analyses except 'known fate' models can discriminate between mortality and permanent emigration, therefore when we speak of survival it is technically 'apparent survival', but during the first season of life we expect no calves to emigrate from the study area. We estimated neonate survival during their first season of life as a function of individual spot traits using Program MARK to analyse complete capture-mark-recapture encounter histories of giraffes first sighted as neonates (White and Burnham 1999). We analysed our encounter histories using Pollock's Robust Design models to estimate age-specific survival (Pollock 1982; Kendall *et al.* 1995), with and without spot covariates, and ranked models using AICc following Burnham and Anderson (2002). We examined linear and quadratic relationships of all 11 spot traits on juvenile survival to determine whether directional, disruptive, or stabilizing selection was occurring. Our study design did not allow for the detection of other forms of selection such as balancing or frequency-dependent selection. Based on previous analyses for this population (Lee *et al.* 2016a, b), we constrained parameters for survival (S) and temporary emigration (γ and γ) to be linear functions of age, and capture and recapture (c and p) were time dependent {(S(A + covariate), γ(A), γ(A), c(t), p(t)}, thus only the spot trait covariates changed among models. Giraffe calf survival does not vary by sex (Lee *et al.* 2016b), so we analysed all calves together. We tested goodness-of-fit in encounter history data using U-CARE (Choquet *et al.* 2009), and we found some evidence for lack of fit (χ^2_{62} = 97, P = 0.01), but we felt this was largely due to lack of age effects in the goodness-of-fit tested model, whereas age effects were included in our model selection and estimation. Additionally, because the computed $\hat{c}$ adjustment was < 3 ($\hat{c}$ = 1.5), we felt our

models fit the data adequately and we did not apply a variance inflation factor (Burnham and Anderson 2002; Choquet *et al*. 2009).

RESULTS

We were able to quantify 11 spot traits using ImageJ, and found the traits with greatest individual heterogeneity as measured by the CV were number of spots and area of spots (these two traits were negatively correlated), and mode shade (**Table 1**). Traits with the least individual variation were solidity and roundness (**Table 1**). Measurements were highly repeatable and reliable with the low variation in measurements from different photos of the same individual (**Table 1**).

We found no spot pattern traits that had significant PO regression coefficients between calves and random cows, but two characters, circularity and solidity (tortuousness) (**Fig 2**) were significantly correlated between calves and their mothers indicating heritability (**Table 1**). PO slope for circularity was 0.52 (SE = 0.16) and for solidity was 0.53 (SE = 0.17). We calculated post-hoc power for our significantly heritable traits of circularity and solidity using our observed effect size (r-squared = 0.25, equivalent to Cohen's $f^2 = 0.33$), alpha level (0.05), and sample size (31), and found power = 0.87. With one-half the observed effect size (r-squared = 0.125) and our same sample size, power = 0.53. To achieve power = 0.80 with r-squared = 0.125, the sample size would need to have been 56 mother-calf pairs.

Our survival analysis of 258 calves first encountered as neonates indicated there was no evidence that individual covariates of spot traits significantly affected survival during the first season of life, but model selection uncertainty was high (**Table 2**). The top-ranked model was the null model of no covariate effects, and all covariate beta coefficient confidence intervals included zero. Power analysis indicated our effective sample size of 944 was far below the 2600 necessary to detect a 0.05 difference in survival probability with a power of 0.80.

DISCUSSION

Our photographic capture-recapture dataset spanning multiple years enabled us to objectively and reliably quantify coat pattern traits of wild giraffes using image analysis software, and demonstrate that giraffe coat pattern traits of spot shape are heritable from mother to calf. The methods we described should serve as a framework for objective quantification of mammalian coat pattern traits, and could also be useful for taxonomic or phenotypic classifications based on photographic coat pattern data. We did not find evidence for fitness consequences of individual variation in spot traits on juvenile survival suggesting that spot pattern traits are currently not under strong directional, disruptive, or stabilizing selection in our study population, but our sample size was insufficient to detect small differences in survival.

Photogrammetry to remotely measure animal traits has used geometric approaches that estimate trait sizes using laser range finders and known focal lengths (Lyon 1994; Lee *et al*. 2016a), photographs of the traits together with a predetermined measurement unit (Ireland et al. 2006; Willisch et al. 2013), or lasers to project equidistant points on animals while they are photographed (Bergeron 2007). We hope the framework we have described using ImageJ to quantify spot characteristics from photographs will prove useful to future efforts at quantifying animal markings, and suggest the resultant trait measurements could be useful in a formal cluster analysis (Kaufman and Rousseeuw 2009) to classify subspecies, polymorphic phenotypes, or other groups based on variation in markings.

Masai giraffe spot patterns are particularly diverse among giraffe subspecies (Dagg 1968), and there are spot patterns in northern Tanzania that bear strong similarities to other giraffe subspecies elsewhere in Africa. Two recent genetic analyses of giraffe taxonomy both placed Masai giraffes as their own species (Brown *et al.* 2007; Fennessy *et al.* 2016), but the lack of quantitative tools to objectively analyze coat patterns for taxonomic classification

may underlie some of the confusion that currently exists in giraffe systematics (Bercovitch *et al.* 2017). We expect the application of image analysis to giraffe coat patterns will provide a new, robust dataset to address taxonomic and evolutionary hypotheses.

Our analyses highlighted two aspects of giraffe spots that were most heritable and which may have adaptive significance. Circularity describes how close the spot is to a perfect circle, and solidity describes how smooth and entire the edges are versus tortuous, ruffled, lobed, or incised. These two characteristics could form the basis for quantifying spot patterns of giraffes across Africa, and gives field workers a new quantitative lexicon for describing spots (**Fig 2**). Our mode shade measurement was a crude metric, and color is greatly affected by lighting conditions, so we suggest standardization of photographic methods to control for lighting if color is to be analyzed in future studies.

One possible explanation for the lack of juvenile survival effects from spot variation in our analysis is the recent reduction in large predator density in our study area (Packer *et al.* 2011; Bauer *et al.* 2015). If the function of the coat markings is to provide anti-predation camouflage, reduced predator densities due to trophy hunting and pastoralist retaliatory killings of predators may have alleviated predation pressure on giraffe calves sufficiently to remove the selection pressure for certain spot traits (Lichtenfeld 2005; Lee *et al.* 2016b). Alternatively, the possibility remains that spot traits may serve adaptive functions such as thermoregulation (Skinner and Smithers 1990), social communication (VanderWaal *et al.* 2014), or indicators of individual quality (Ljetoff *et al.* 2007), rather than anti-predation camouflage, and thus may demonstrate associations with other components of fitness, such as survivorship in older age classes or fecundity. Individual recognition, kin recognition, and inbreeding avoidance also could play a role in the evolution of spot patterns in giraffes (Beecher 1982; Tibbetts and Dale 2007; Sherman *et al.* 1997). Different aspects of spot traits

may also be nonadaptive and serve no function, or spot patterns could be affected by pleiotropic selection on a gene that influences multiple traits (Lamoreux *et al.* 2010).

Our study revealed that several spot traits are heritable and, hence, able to respond to selection in giraffe populations. However, given that these spot characteristic traits did not significantly affect neonatal survival, the main theory for the adaptive nature of giraffe spots (Langman 1977; Mitchell and Skinner 2003), we conclude that spot patterns are currently of minor importance for the adaptation of our study population, although this may be a recent consequence of fewer predators. It may also be that some patterns affect fitness only in certain environments, or our measurements of spot traits may not accurately reflect the aspects of spot patterns most important to adaptation for neonatal crypsis. Other aspects of spot variation may prove to be more relevant to fitness, such as social effects of individual recognition or kin recognition, or thermoregulation, and deserve further investigation. We may also have simply lacked sufficient sample sizes necessary to detect small adaptation or fitness effects. Small and very difficult to detect differences in fitness may have profound influence on phenotypic evolution compounded over thousands of generations (Orr 2009).

Patterned coats of mammals are hypothesized to be formed by two distinct processes: a spatially oriented developmental mechanism that creates a species-specific pattern of skin cell differentiation and a pigmentation-oriented mechanism that uses information from the pre-established spatial pattern to regulate the synthesis of melanin (Eizirik *et al.* 2010). The giraffe skin has more extensive pigmentation and wider distribution of melanocytes than most other animals (Dimond and Montagna 1976). Coat pattern variation may reflect discrete polymorphisms potentially related to life-history strategies, a continuous signal related to individual quality, or a combination of both. Future work on the genetics of coat patterns will hopefully shed light upon the mechanisms and consequences of coat pattern variation.

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DATA AVAILABILITY

We have deposited the primary data underlying these analyses as follows:

- Sampling locations, original data photos, and spot trait data: Dryad

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FIGURE CAPTIONS

Fig 1. Representative images of spot patterns of mother-calf pairs of Masai giraffes (Giraffa camelopardalis tippelskirchii) from the Tarangire ecosystem, Tanzania used in this study. The blue rectangle shows the area analysed using ImageJ to characterize spot pattern traits.

Fig 2. Representative spot outlines from Masai giraffes in northern Tanzania and their corresponding circularity and solidity values. Ranges of spot trait values from 213 calves are given in parentheses.

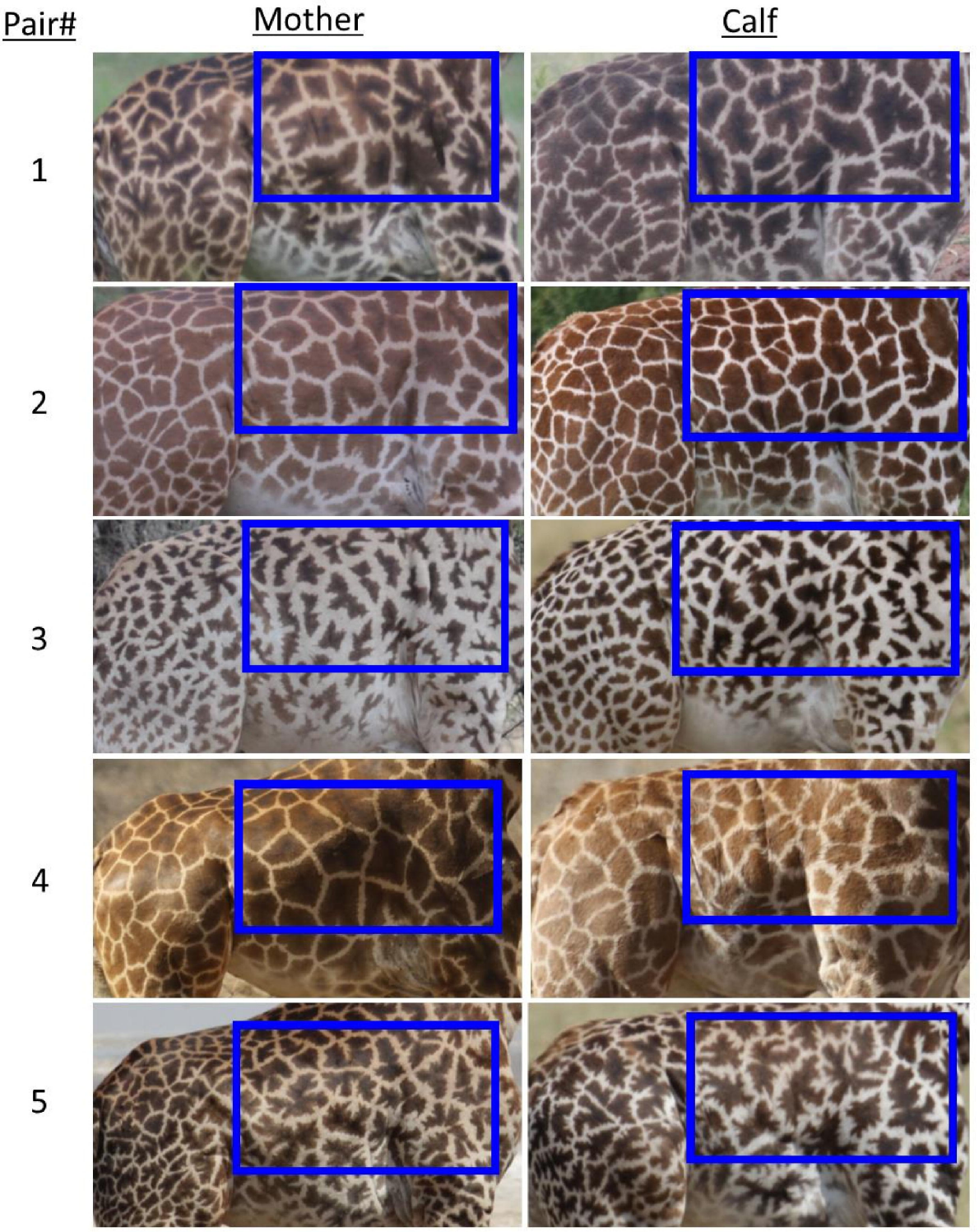
Table 1. Summary statistics for parent-offspring regressions of spot traits of Masai giraffes in northern Tanzania. Mean trait values, SD (standard deviation), CV (among-individuals coefficient of variation), Repeatability (mean % variation in measurements from different pictures of the same individual), PO slope coefficients (represents one-half of total heritability), F-statistics, P values, and r-squared values are provided. Significantly heritable traits are in bold.

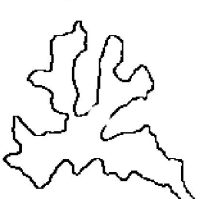
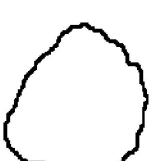
	Number	Area	Perimeter	Angle	**Circularity**	Maximum Caliper	Feret Angle	Aspect Ratio	Roundness	**Solidity**	Mode Shade
Mean	18.9	0.04	0.99	87.96	0.51	0.29	88.2	1.69	0.63	0.84	6924050
SD	7.5	0.01	0.25	15.39	0.08	0.06	14.5	0.15	0.04	0.04	3930565
CV	0.40	0.39	0.25	0.17	0.15	0.19	0.16	0.09	0.06	0.05	0.57
Repeatability	11	11	13	4	9	8	7	5	3	2	13
PO Slope Coefficient	0.20	0.20	0.27	0.04	**0.52**	0.21	-0.15	0.19	0.08	**0.53**	0.44
PO Coefficient SE	0.23	0.21	0.18	0.20	**0.16**	0.21	0.15	0.18	0.17	**0.17**	0.22
$F_{1,29}$	0.76	0.87	2.27	0.04	**9.97**	1.01	0.91	1.11	0.19	**9.73**	4.16
P value	0.39	0.36	0.14	0.84	**0.0037**	0.32	0.35	0.30	0.66	**0.0041**	0.051
r-squared	0.03	0.03	0.07	0	**0.26**	0.03	0.03	0.04	0.010	**0.25**	0.13

Table 2. Model selection results for giraffe calf survival as a linear or quadratic function of spot trait covariates. The top-ranked model was the null model of no covariate effects, and all covariate beta coefficient confidence intervals included zero, indicating no evidence for significant spot trait effects on calf survival. Model structure in all cases was {S(A + Covariate) g”(A) g’(A) p(t) c(t)} with covariate structure in survival. Minimum AICc = 3247.45, W = AICc weight, k = number of parameters.

Covariate Model	ΔAICc	W	k
Null (no spot trait covariate)	0	0.15	45
Aspect Ratio	0.96	0.09	46
Roundness	1.12	0.08	46
Solidity	1.50	0.07	46
Number of Spots	1.79	0.06	46
Angle	1.90	0.06	46
Perimeter	1.98	0.05	46
Circularity^2	2.06	0.05	47
Feret Angle^2	2.10	0.05	47
Mode Shade	2.11	0.05	46
Maximum Caliper	2.30	0.04	46
Feret Angle	2.31	0.04	46
Circularity	2.32	0.04	46
Solidity^2	2.49	0.04	47
Angle^2	2.84	0.03	47
Number of Spots^2	3.11	0.02	47

Aspect Ratio[2]	3.18	0.02	47
Roundness[2]	3.32	0.02	47
Mode Shade[2]	3.41	0.02	47
Maximum Caliper[2]	3.71	0.01	47
Perimeter[2]	4.53	0.01	47


Pair#
Mother
Calf
1
2
3
4
5

Shape	Circularity (0.10—0.94)	Solidity (0.52—0.96)
	0.15	0.66
	0.13	0.60
	0.88	0.94
	0.37	0.76
	0.40	0.83
	0.57	0.82
	0.44	0.76
	0.12	0.53
	0.83	0.96