MECHANISMS MEDIATING LIFE HISTORY TRAITS IN THE SPOTTED HYENA (*CROCUTA CROCUTA*)

By

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ABSTRACT

MECHANISMS MEDIATING LIFE HISTORY TRAITS IN THE SPOTTED HYENA (*CROCUTA CROCUTA*)

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My dissertation focuses on understanding the mechanisms underpinning growth, reproduction, and survival in the spotted hyena. Following a general introductory chapter, my dissertation is composed of four independent research chapters. I begin with Chapter 2 in which my colleagues and I document a positive linear relationship between social dominance rank and telomere length. We also report significant variability in telomere length of high-ranking females among different social groups, suggesting that both social dominance rank and group membership influence this important biomarker of aging. In Chapter 3, we describe the role of juvenile concentrations of the hormone, insulin-like growth-factor -1 (IGF-1), in predicting trade-offs between early-life growth and later-life reproduction and survival among female hyenas. In Chapter 4, I explore IGF-1 as a potential mechanism of female-biased sexual size dimorphism by documenting sex-biased concentrations, sensitivities, and adaptive values of IGF-1 during the early postnatal period. Finally, in Chapter 5, I describe that age-related improvement and senescence in reproductive performance varies with social dominance rank among female hyenas. Cumulatively, my dissertation is an exploration of how physiological mechanisms may be used to understand social, physiological, and evolutionary forces operating in a free-living social carnivore. My work offers a unique contribution to the field of life-history evolution and furthers our understanding of the mechanisms that give rise to it.

I dedicate this thesis to my late father, John R. Lewin.

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Ph.D. I could not have been able to cross this finish line without your love. Thank you.

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CHAPTER 1: GENERAL INTRODUCTION

General Introduction

Life-history theory seeks to explain the diverse patterns in reproduction and survival exhibited by organisms. The increased documentation of natural history patterns observed within and among species has led to the construction of evolutionary theoretical frameworks to explain common "strategies." These strategies are often conceptualized along a fast-slow continuum with "fast-living" strategies marked by faster development, higher initial reproductive rates, and shorter lifespans, and "slow-living" strategies described by slower development, lower reproductive rates, and longer lifespans (Western & Ssemakula 1982; Promislow & Harvey 1990; Stearns 1992). Fundamentally, each strategy is governed by the optimization of energy, with the balance of resources tipped towards maximizing fitness. In species organized by linear dominance hierarchies, an individual's social rank largely determines its growth and reproductive fitness relative to its peers. While high-ranking individuals appear to exhibit a "faster" pace of life and low-ranking individuals a "slower" pace of life, little research has explored if and/or how social rank may contribute towards the evolution of life-history strategies exhibited within a population or species.

In this dissertation, I have attempted to describe the evolutionary and physiological mechanisms underpinning life history variation in a social mammal, the spotted hyena (*Crocuta crocuta*). Within a social group, called a clan, high-ranking females grow faster, wean and start reproducing at earlier ages, and exhibit higher reproductive rates than do lower-ranking females. Interestingly, high-ranking females do not appear to suffer from shorter lifespans, which would be expected by a "fast-living" strategy. My research has focused on understanding the mediation of this apparent contradiction with the hope that by integrating the seemingly disparate worlds of evolutionary biology and biogerontology (the study of aging mechanisms), we may better understand how mechanisms of life histories have evolved across social and environmental gradients.

Summary of Chapter 2

Presumably, faster growth and higher reproductive rates are linked to shorter lifespans through the erosion of physiological functions from accumulating cellular "wear and tear." Much like a bank account with a fixed amount of funds and increasing withdraws, the "disposable soma" theory predicts that the degradation of physiological functionality (senescence) is a realized consequence of when an organism's energetic cache is no longer sustainable (Kirkwood & Holliday 1979; Kirkwood & Rose 1991). By occupying the top positions in a clan, high-ranking spotted hyenas enjoy priority of access to key resources and receive far fewer acts of aggression. My collaborators and I hypothesized that these social benefits may offset any accumulation of cellular stress typically associated with faster growth and higher reproductive rates. We also hypothesized that this relationship may vary among clans that experience different ecological conditions.

We tested these hypotheses by measuring telomere length among adult hyenas occupying various social rank positions among five different clans in the Masai Mara National Reserve of southwestern Kenya. Telomeres are highly conserved, noncoding, repetitive DNA sequences that confer chromosomal stability and buffer against degradation of coding sequences (Greider & Blackburn 1985; Harley, Futcher & Greider 1990). When telomeres shorten to a critical length a regulatory cascade is unleashed often culminating in DNA damage ("wear and tear") and replicative senescence (Monaghan & Haussmann 2006). Thus, shorter telomere lengths have been viewed as useful biomarkers of accumulated cellular stress. We found a strikingly positive linear relationship between telomere length and social dominance rank, with high-ranking females exhibiting the longest telomeres and low-ranking males exhibiting the shortest (Lewin *et al.* 2015). We also found variable telomere lengths in high-ranking females among the different clans independent of local food abundance, suggesting that other socioecological variables may be at play (Reznick *et al.* 2004). Our results confirmed

our hypothesis that high-ranking females do not appear to incur large degrees of cellular damage despite high energetic withdraws made for growth and reproduction.

Summary of Chapter 3

The theory of antagonistic pleiotropy predicts that a shared mechanism may have opposing effects on different life-history traits, such that a beneficial trait earlier in life may have an inextricable cost associated with it later in life (Williams 1957; Rose 1985). High-ranking female hyenas seem to reap many early-life benefits (e.g. fast growth, early age at first reproduction) without incurring many later-life costs (e.g. short telomere lengths, short lifespans) so we investigated whether or not they are subject to pleiotropic mechanisms. The insulin-like growth-factor-1 (IGF-1) is a metabolic hormone that is hypothesized as a pleiotropic agent in life-history variation due to its well-documented effects in laboratory model organisms (Dantzer & Swanson 2012) and some wild vertebrates species (Sparkman, Vleck & Bronikowski 2009). Because IGF-1 confers its first benefit as growth we expected concentrations of IGF-1 during this period to predict later-life costs.

We found that juvenile concentrations of IGF-1 predicted heavier juvenile mass, which in turn predicted greater survival to reproductive maturity. However, independent of mass, higher juvenile concentrations of IGF-1 predicted earlier age at first parturition and reduced longevity in adulthood. Our results highlight the importance of early postnatal development as a determination point in mammals, and suggests that concentrations of IGF-1 during this sensitive period can be used to predict important later-life trade-offs between growth, reproductive fitness, and lifespan in wild, long-lived animals.

Summary of Chapter 4

In the previous chapter, we found higher concentrations of IGF-1 among heavier juvenile female hyenas. The strong relationship between mass and IGF-1 led us to investigate the role of IGF-1 as a potential mediator of female-biased sexual size dimorphism (SSD) among spotted hyenas. Here, we had three goals. The first was to describe intra- and inter-sexual variation in

circulating IGF-1 concentrations across the lifespan. If IGF-1 mediates the emergence of SSD in spotted hyenas, then we should expect sex differences in circulating concentrations starting before the dimorphic growth period between 1-4 years of age (Swanson *et al.* 2013). In many wild vertebrate species, sex-biased sensitivity to extrinsic variables during postnatal development is believed to contribute largely to the realized difference, or degree, of SSD observed in adulthood (Post *et al.* 1999; Toïgo, Gaillard & Michallet 1999; Badyaev, Whittingham & Hill 2001; Wilkin & Sheldon 2009). Thus, our second goal was to identify whether sex-biased sensitivities to various socio-ecological variables affected juvenile IGF-1 concentrations in both sexes. Theoretically, SSD may arise when the selective optimum for growth or size differs between the sexes (Ralls 1977; Lande 1980; Price 1984). As such, our third goal was to investigate whether sex-biased fitness effects existed among spotted hyenas. Our results showed that females displayed higher concentrations of IGF-1 than did males, starting before and lasting beyond the dimorphic growth period. We also found that socioecological variables predicted variation in IGF-1 among juvenile males but not among juvenile females. Lastly, we found that juvenile mass predicted survival to reproductive maturity among females but not among males, suggesting a selective force by which SSD may be maintained in this species.

Summary of Chapter 5

In many hierarchical societies, high-ranking individuals enjoy greater reproductive fitness than low-ranking individuals. Previous work by Holekamp et al. (1996) found that adult, high-ranking female hyenas also benefit from higher social status as evidenced by more successful performance on a number of reproductive traits. In this chapter, we sought to describe patterns of rank-related reproductive performance over the lifespan. We specifically tested two non-mutually exclusive hypotheses used to predict age-related improvement and decline in reproductive performance using 27 years of individual-based, longitudinal data from the MSU Mara Hyena Project, started by Drs. Kay Holekamp and Laura Smale. We found that female

hyenas exhibit both reproductive improvement and senescence and that the patterns vary with social rank. Higher-ranking females started reproducing at younger ages and displayed higher reproductive success than lower-ranking females. However, these females typically did not live as long as lower-ranking females, suggesting a potential life-history trade-off. Some lower-ranking females lived exceptionally long lives, which afforded them a level of reproductive fitness comparable to that of high-ranking females. Interestingly, alpha females seemed unaffected by any potential trade-offs. Specifically, two alphas females started reproducing at young ages with high reproductive success and exhibited longer than average lifespans. The findings of this chapter offer a unique and rarely documented perspective on how social dominance rank may influence population dynamics.

CHAPTER 2: SOCIOECOLOGICAL VARIABLES PREDICT TELOMERE LENGTH IN WILD SPOTTED HYENAS

Nora Lewin, Lisa A. Treidel, Kay E. Holekamp, Ned J. Place, and Mark F. Haussmann
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Abstract

Telomeres are regarded as important biomarkers of ageing and serve as useful tools in revealing how stress acts at the cellular level. However, the effects of social and ecological factors on telomere length remain poorly understood, particularly in free-ranging mammals. Here, we investigated the influences of within-group dominance rank and group membership on telomere length in wild adult spotted hyenas (*Crocuta crocuta*). We found large effects of both factors; high-ranking hyenas exhibited significantly greater mean telomere length than did subordinate animals, and group membership significantly predicted mean telomere length within high-ranking females. We further inquired whether prey availability mediates the observed effect of group membership on telomere length, but this hypothesis was not supported. Interestingly, adult telomere length was not predicted by age. Our work shows for the first time, to the best of our knowledge, the effects of social rank on telomere length in a wild mammal and enhances our understanding of how social and ecological variables may contribute to organismal senescence.

Introduction

Social and ecological factors can have important effects on animal health, yet the proximate mechanisms mediating such effects are still being determined. Within the past decade, telomere length has been identified as a candidate mechanism through which individual fitness and life expectancy may vary (Monaghan & Haussmann 2006). Telomeres are highly conserved, noncoding, repetitive DNA sequences that cap the ends of eukaryotic chromosomes, confer chromosomal stability, and buffer against degradation of coding sequences following semiconservative DNA replication (Harley *et al.* 1990). When telomeres shorten to a critical length, a regulatory cascade is triggered, potentially culminating in DNA damage and cell death (Harley *et al.* 1990). Hence, telomeres may figure importantly in organismal ageing and the mediation of life history variation (Monaghan & Haussmann 2006).

Although telomeres tend to shorten with age (Harley *et al.* 1990; Hall *et al.* 2004; Monaghan & Haussmann 2006), the rate at which this happens may be influenced by the quality of an organism's environment (Monaghan & Haussmann 2006; Angelier *et al.* 2013; Mizutani *et al.* 2013). In animal societies structured by linear dominance hierarchies, social rank can act as a filter through which ecological factors affect the individual. An individual's dominance status often determines its priority of access to key resources and has important downstream effects on health (Sapolsky 2005). Previous work in despotic animal societies has shown large effects of social rank on stress physiology (Cavigelli & Chaudhry 2012), but we know nothing about its influence on telomere length. Here, we tested predictions of an hypothesis suggesting that telomere length varies with socioecological conditions experienced by adult free-living spotted hyenas (*Crocuta crocuta*). Specifically, we inquired whether leucocyte telomere length varies with dominance status within or among hyena groups when individuals experience more or less challenging ecological conditions.

Materials and Methods

Study species

Spotted hyenas are large-bodied, long-lived carnivores that live in groups called clans, which may contain over 100 members (Holekamp *et al.* 2012). Spotted hyenas prey mainly on large and medium-sized ungulates they kill themselves (Kruuk 1972). Clan-mates defend a common group territory (Kruuk 1972). Clans are structured by rigid linear dominance hierarchies in which an individual's rank position determines its priority of access to food, thereby influencing virtually every aspect of its biology (Holekamp *et al.* 2012). In this species, dominance rank is acquired via associative learning at a young age and maintained through low-intensity, ritualized aggressive behaviors (Smale, Frank & Holekamp 1993). Female spotted hyenas are the more aggressive sex, and are socially dominant to all adult males not born in the clan. Females are philopatric, whereas males typically disperse from their natal clans after puberty, which occurs at around 24 months of age. Males disperse most commonly at 2-5 years

of age (Holekamp & Smale 1998; Van Horn, McElhinny & Holekamp 2003). Males may take several months to become successfully integrated into a new clan; immigrant males are subordinate to all natal animals and to immigrant males with longer tenure in the new clan (Holekamp & Smale 1998; East & Hofer 2001). It is rare for males to remain within their natal clans long after sexual maturity, so we were only able to obtain four telomere samples from three adult natal males.

Data collection

We measured telomere length in 64 whole blood samples drawn from 54 adult hyenas. For 9 individuals, we had repeated samples taken at different ages during adulthood (Table A.1). Ages at sampling ranged from 2.5 to 13 years, based on known birthdates (± 7 days) or tooth wear data (± 6 months) (Van Horn *et al.* 2003). Adult hyenas were members of 5 social groups, called clans, sampled between 1999 and 2013 in the Masai Mara National Reserve, Kenya (Table A.2). All clans contained multiple matrilines comprised of females and their descendants, as well as multiple immigrant males. Among both natal females from different matrilines and male immigrants, within-clan relatedness is effectively zero (Van Horn *et al.* 2004).

The habitat occupied by all 5 clans was primarily open, rolling grassland with permanent water and year-round prey availability. Local prey density was calculated as the mean number of ungulates counted within 100 meters of multiple 4-km transect lines run through each territory at biweekly intervals for the 2 years prior to collection of each blood sample. Local prey density was available for only 1 year prior to collection for one high-ranking female from the Fig Tree clan and two high-ranking females from the Mara River clan. Per capita prey abundance was calculated as local prey density divided by the mean number of adults present within the respective clan during the 2 years prior to collection of each blood sample. Adults included all natal females, males, and immigrant males. Within each clan, dominance rank matrices were constructed based on outcomes of dyadic agonistic interactions (Smale *et al.* 1993). We

standardized rank to account for variation in clan size; a relative rank of 1 was the highest-ranking animal in the clan, and the individual with a relative rank of -1 was the lowest-ranking. High-ranking females were considered those with relative ranks of 1 to 0, and low-ranking females were considered those with relative ranks of -0.01 to -1 at the time of darting. Hyenas were immobilized using Telazol (Zoetis, South Bend, IN; 6.5 mg/kg) administered via a lightweight plastic dart fired from a CO_2-powered rifle (Telinject Inc., Saugus, CA). Blood samples were collected from the jugular vein into EDTA-treated vacutainers and promptly frozen in liquid nitrogen until they could be shipped on dry ice to Michigan State University where they were stored at -80˚C.

Telomere analysis

Genomic DNA was extracted using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA) and kept frozen at -80°C until telomere analysis. Once shipped on dry ice to Bucknell University, DNA was digested overnight with *HinfI* and *Rsa*I, and separated using pulsed field gel electrophoresis (3 V cm^{-1}, 0.5-7.0 s switch times, 14˚C) for 21 hours, followed by in-gel hybridization at 37˚C overnight with a radioactive-labelled telomere-specific oligo $(CCCTAA)_4$. Gels were then placed on a phosphorscreen (Amersham Biosciences, Buckinghamshire, UK), which was scanned on a Storm 540 Variable Mode Imager (Amersham Biosciences). Position and strength of the radioactive signal were determined by densitometry using IMAGEQUANT 5.03v and IMAGEJ 1.42q. Mean telomere length was calculated using the formula: $L = \Sigma(OD_i \times L_i)/ \Sigma(OD_i)$ where OD_i is the densitometry output at position *i* and L_i is the length (in basepairs) of the DNA at position *i*. For all samples, DNA integrity was evaluated empirically by a nucleic acid stain following agarose gel electrophoresis. DNA was deemed suitable for TRF length analysis if it was characterized as “a single compact crown-shaped band that migrates in parallel with the other samples on the gel” (Kimura *et al.* 2010). Two control hyena samples were run twice in each gel to determine intra- (2.79%) and inter-assay variability (3.16%).

Statistics

We used a Shapiro-Wilk normality test to confirm that the data were normally distributed (W = 0.99, p = 0.79). We then ran a linear mixed effects model (LMM) of restricted maximum likelihood to assess the effects of dominance rank and age on adult mean telomere length using the lme4 package (Bates *et al.* 2014) in R v.3.1.1 (R Development Core Team 2014). We included random effects of clan membership, sampling year, and ID because we sampled among 5 different clans, across multiple years, and had repeated measures on 9 hyenas. Clan membership and ID, but not sampling year, significantly improved the fit of the model based on likelihood ratio tests ($P < 0.05$) and were included in the final LMM. We further tested for effects of clan membership, prey density, and age on telomere length using only samples from high-ranking females. This allowed us to control for dominance rank while retaining a large enough sample size for analysis. Neither random effect of ID nor sampling year improved the model so we employed a multiple linear regression for our analysis. Pairwise comparisons among clans were calculated by least square means and Satterwaithe's approximation for degrees of freedom (West & Welch 2014). To identify whether there was an age effect on telomere length, we ran a LMM with ID as a random effect for individuals that were sampled repeatedly as adults. We employed a simple regression model to investigate whether maternal telomere length predicted telomere length in adult offspring. Here mothers and offspring with multiple samples were assigned a single mean adult telomere length.

Results

Among all adults, relative dominance rank significantly predicted telomere length ($F_{1, 34.9}$ = 8.43, P = 0.006; Figure 2.1), but age did not ($F_{1, 53.5}$ = 0.76, P = 0.39). The random effects for hyena ID and clan explained 26.8% and 24.6%, of the residual variation, respectively, and significantly improved the fit of the model (ID: χ^2 = 4.83, df =1, P = 0.028; clan: χ^2 = 4.24, df = 1, P = 0.039). Among high-ranking females (n= 30 telomere samples from 26 hyenas), only clan membership significantly predicted telomere length (Table 2.1; Figure 2.2). For 9 individuals

with repeated sampling, the random effect of ID explained 58.7% of the residual variation and improved the fit of the model (χ^2 = 4.71, df =1, P = 0.030), but there was no significant effect of age on telomere length ($F_{1, 13.7}$ = 1.57, P = 0.20). We also found no relationship between telomere lengths in mothers and their adult offspring (R^2 = 0.11, P = 0.39; Table A.3).

Table 2.1. (a) Results from multiple regression model predicting telomere length among high-ranking females only (n = 30 telomere samples from 26 hyenas). The Talek clan is set as the reference category. Bolded *P*-values indicate statistically significant differences at an alpha = 0.05.

(a)

fixed effect	estimate	s.e.	*t*-value	*P*-value
intercept	13.91	0.67	20.86	**<0.001**
Fig Tree	0.15	0.88	0.17	0.863
Mara River	-2.27	0.79	-2.87	**0.009**
Serena N.	-2.36	0.93	-2.55	**0.018**
Serena S.	-1.77	0.66	-2.68	**0.013**
prey per hyena	0.26	0.18	1.43	0.167
age	-0.03	0.08	-0.46	0.647

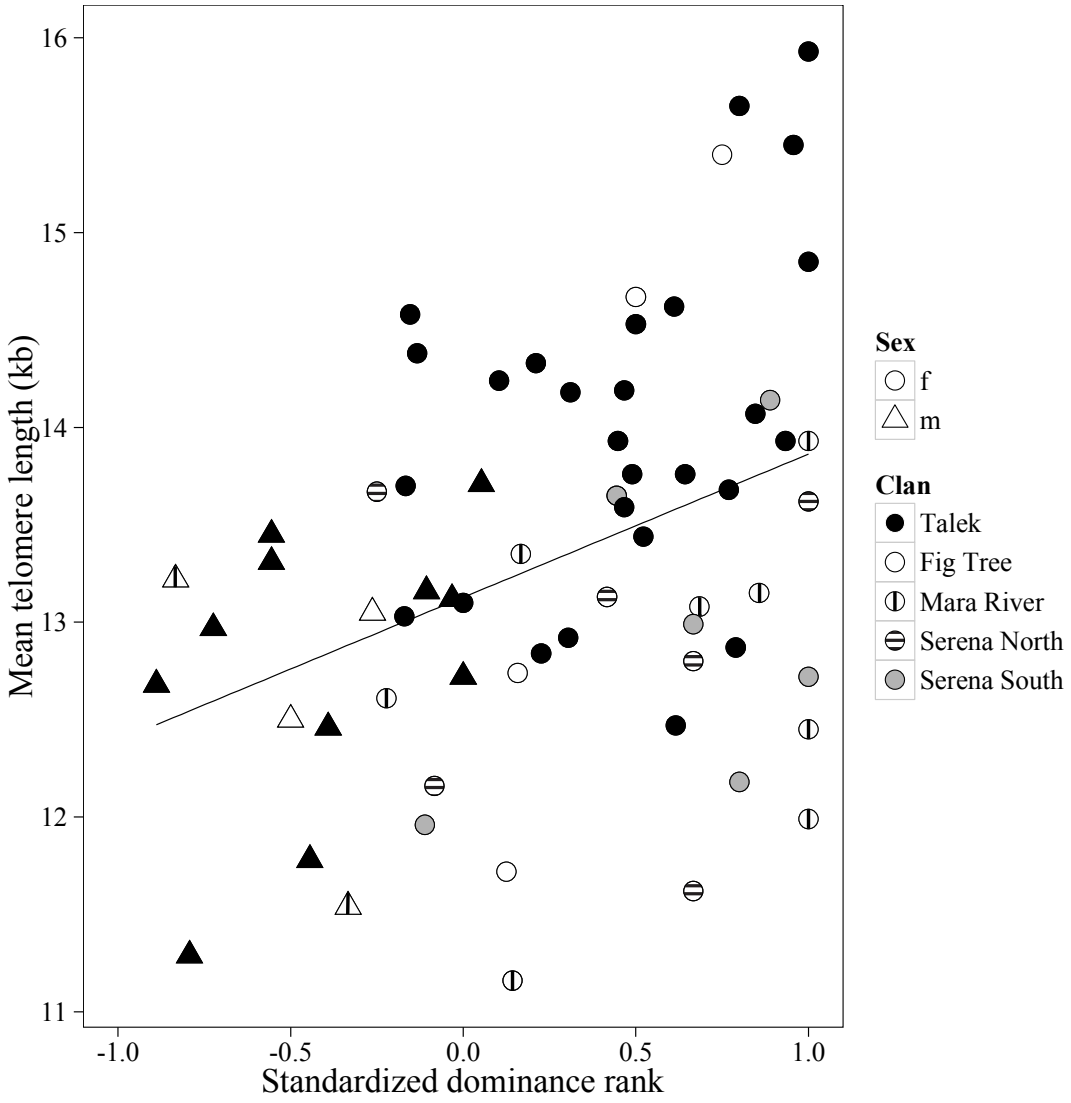

Figure 2.1. Mean telomere length in relation to relative dominance rank in adult hyenas. Regression line shown is from a linear mixed-effects model with all clans included. A dominance rank of 1 represents the highest-ranking animal within a clan at the time of sampling, and the individual with a dominance rank of -1 represents the lowest-ranking. Symbol pattern denotes the clan to which each individual belonged. Circles: females; triangles: males.

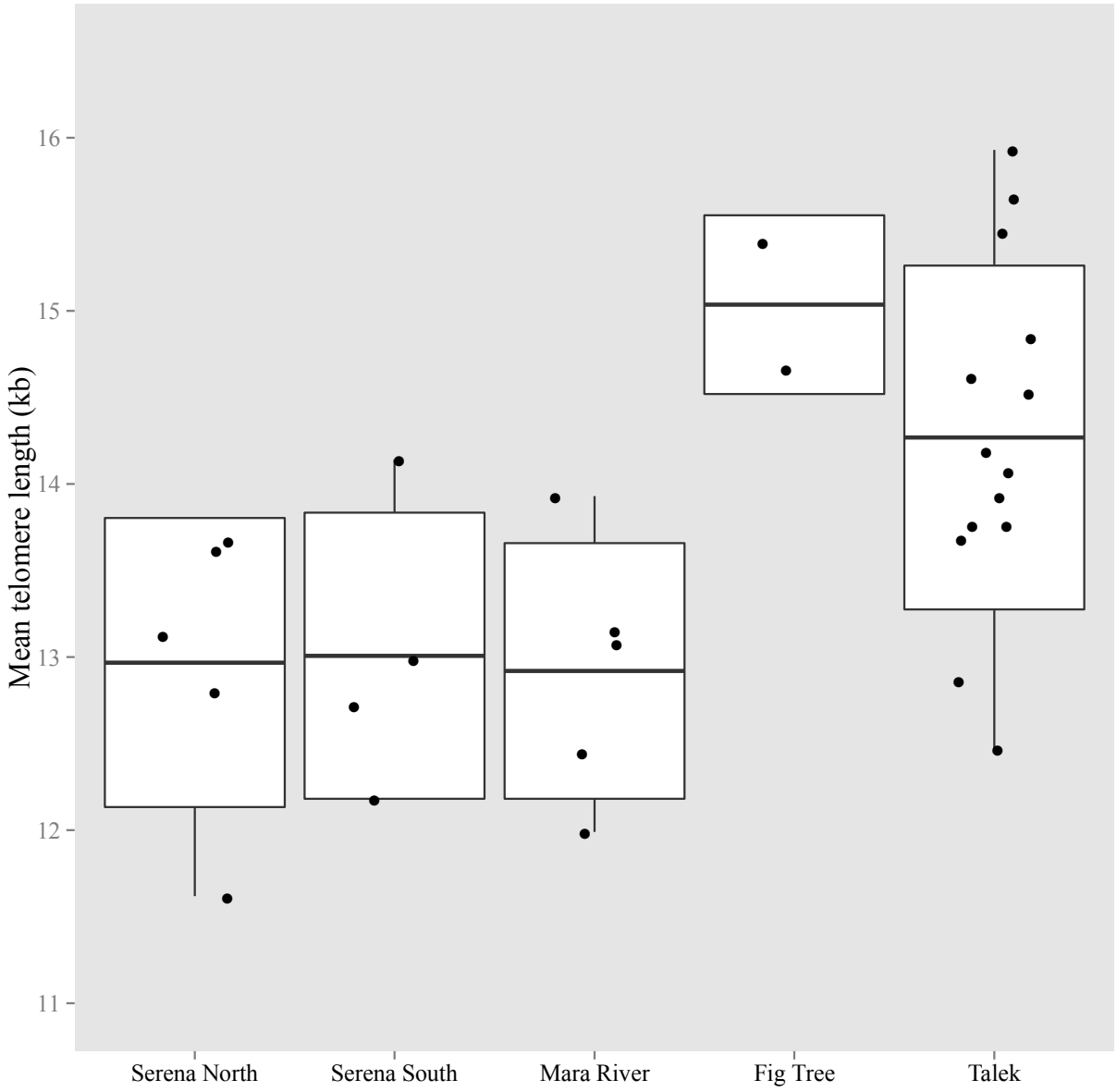

Figure 2.2. Mean telomere length among high-ranking females from 5 clans. Box plots show group mean (line within box) ± s.d. (box) and range (whiskers).

Discussion

In hyena societies, high social rank ensures superior food access at kills; this forces many low-ranking animals to forage near territory edges or commute great distances in search of uncontested food (Hofer & East 1993a; Boydston *et al.* 2005). Low-ranking female and male hyenas may thus incur high maintenance costs, similar to birds subjected to environmental challenges (Hall *et al.* 2004; Angelier *et al.* 2013; Mizutani *et al.* 2013). Metabolic stress may cause high concentrations of reactive oxidative species and stress hormones, which are known correlates of telomere shortening (Haussmann & Marchetto 2010). In spotted hyenas in Tanzania, dominance rank predicted circulating glucocorticoid concentrations among non-lactating females, with lower concentrations found in high- than low-ranking females (Goymann *et al.* 2001). Our finding that high-ranking hyenas display longer telomeres than low-ranking hyenas is consistent with earlier work, and further suggests that social subordination has important consequences at the cellular level.

Age was not a significant predictor of telomere length in any of our analyses and appears not to affect telomere length strongly among adult hyenas. Although counterintuitive, this is consistent with findings in long-lived birds, where the rate of telomere shortening is notably slower during adulthood than early development (Hall *et al.* 2004; Monaghan & Haussmann 2006). Hyenas from high-ranking matrilines might be born with longer telomeres than those from low-ranking matrilines, but we could not test this, nor did we find any evidence that maternal telomere length predicts offspring telomere length (Table A.3). Increasing evidence shows that pre- and post-natal stress can have long-lasting effects on adult telomere length (Hall *et al.* 2004; Haussmann *et al.* 2012; Herborn *et al.* 2014). Spotted hyenas experience the highest mortality rates during the period immediately after weaning (Watts *et al.* 2009), and this may coincide with a high rate of telomere shortening. We speculate that the rank-related variation observed in adult telomere length may have early developmental origins.

As a random effect, clan membership explained a large proportion of the residual variance in telomere length among adult hyenas. We investigated the clan effect further by modeling it as a fixed effect where it significantly predicted telomere length among high-ranking females. Within-clan relatedness among spotted hyenas averages around zero (Van Horn *et al.* 2003), allowing us to rule out clan-specific genetic effects. Therefore, we tested the hypothesis that telomere length might be influenced by local prey availability, which varies among clan territories (Table 2.1). However, this was not a significant predictor within high-ranking females (Table 2.1), implying that other factors that vary among clans are likely in play. Our analysis also revealed pronounced individual variation in telomere length even though sampled females all occupied top positions in their respective dominance hierarchies. Thus, there appears to be considerable inter inter- and intraclan variation in the relationship between dominance rank and telomere length. This is the first study to investigate the influence of social dominance on telomere length in a non-human species, and we encourage future experimental testing of this relationship in other species.

APPENDIX

Supplementary Material for Chapter 2

Table A.1. Information on repeated samples from the same individuals.

	Hyena ID	Clan	Age1 (yr)	Age2 (yr)	Age3 (yr)
1.	ali	Talek	3.93	9.24	-
2.	eco	Mara River	5.02	5.32	-
3.	guci	Talek	3.43	6.51	-
4.	mrph	Talek	2.72	3.72	-
5.	bail	Talek	7.91	11.28	-
6.	hex	Talek	4.29	7.42	-
7.	nav	Talek	6.45	9.71	13.04
8.	ldv	Talek	3.50	7.57	-
9.	oak	Talek	2.52	7.84	-

Table A.2. Information on the demography and territories of sampled clans.

	Talek	Serena North	Mara River	Serena South	Fig Tree
years sampled	1999-2013	2012-2013	2002-2009	2012-2013	2006-2009
mean territory size	57.3 km^2	50 km^2	31 km^2	28 km^2	52.91 km^2
mean prey density per adult hyena	2.46 animals/ hyena	6.48 animals/ hyena	5.22 animals/ hyena	4.23 animals/ hyena	4.88 animals/ hyena

Table A.3. Results from a simple linear regression of offspring adult telomere length on maternal telomere length (df = 7).

	estimate	s.e.	*t*-value	*p*-value	R^2
intercept	11.71	2.82	4.16	0.004	
maternal telomere length	0.17	0.19	0.93	0.39	0.11

CHAPTER 3: JUVENILE CONCENTRATIONS OF IGF-1 PREDICT LIFE-HISTORY TRADE-OFFS IN A WILD MAMMAL

Nora Lewin, Eli M. Swanson, Barry L. Williams, and Kay E. Holekamp
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Introduction

Life history theory posits that realized phenotypic outcomes represent compromises among fitness costs and benefits (Stearns 1992). Trade-offs result when an increase in fitness due to one trait occurs concomitantly with negative changes in fitness associated with another trait (Stearns 1989, 1992; Promislow & Harvey 1990; Kirkwood & Rose 1991; Zera & Harshman 2001). Selection can act at different times during the lifespan so fitness costs of a trait need not accrue at the same time as its benefits, resulting in age-structured trade-offs. Such trade-offs are hypothesized to contribute importantly to the diversity of observed life histories (Promislow & Harvey 1990; Kirkwood & Rose 1991; Stearns 1992; Metcalfe & Monaghan 2003; Roff & Fairbairn 2007). Despite great variation in life histories, some trade-offs appear to occur very frequently in nature and across taxa (Western & Ssemakula 1982; Stearns 1983; Promislow & Harvey 1990; Bielby *et al.* 2007; Swanson & Dantzer 2014; Lemaître *et al.* 2015). For instance, life-history strategies are often conceptualized along a fast-slow continuum. Initial costs and delayed benefits (e.g. slow development, low reproductive rate, and long lifespan) characterize a "slow" pace of life, whereas the converse, initial benefits and delayed costs, characterizes a fast pace of life. In fluctuating or competitive environments where no single life history is optimal, both fast and slow paces may occur within a population (Descamps *et al.* 2006; Nussey *et al.* 2008b; Sparkman *et al.* 2009; Schuett *et al.* 2015; Huchard *et al.* 2016). However, it remains largely unclear how and when an organism's life history is determined.

In the past two decades, there has been considerable interest in identifying the physiological mechanisms mediating life-history variation (Zera & Harshman 2001; Ricklefs & Wikelski 2002; Flatt & Heyland 2011; Dantzer & Swanson 2012; Swanson & Dantzer 2014). Endocrine pathways are hypothesized to facilitate life-history evolution through their ability to affect multiple traits concurrently while also remaining relatively plastic in their responses to immediate environmental cues, such as food abundance or social pressures (Ketterson & Nolan Jr 1992; Dufty, Clobert & Møller 2002; Ricklefs & Wikelski 2002; Flatt & Heyland 2011).

Regardless of their intrinsic trade-offs, all life histories theoretically optimize the allocation of limited energy resources. Therefore metabolic hormones may play significant intermediary roles by signaling appropriate distribution of energy in response to current environmental conditions. This mechanism should theoretically operate during a sensitive period in early in life, when the organism passes through a phase of development critical to the determination of resultant life histories (Ketterson & Nolan Jr 1992; Lindstrom 1999; English *et al.* 2016). Thus, early hormone concentrations may serve as important agents of evolution by organizing the development of a life history best suited for the predicted adult environment.

The polypeptide hormone, insulin-like growth factor-1 (IGF-1), has been proposed as one candidate mechanism for shaping life-history traits due to its critical importance during development and its pleiotropic effects throughout the lifespan (Tatar, Bartke & Antebi 2003; Kenyon 2005; Dantzer & Swanson 2012; Bartke, Sun & Longo 2013; Swanson & Dantzer 2014). IGF-1 and its associated pathways are well characterized in both vertebrate and invertebrate taxa. In vertebrates, the liver primarily produces IGF-1, but almost all tissues synthesize IGF-1 as well, enabling IGF-1 to have both endocrine and paracrine effects (Bartke *et al.* 2013). Work with laboratory animal models has shown that high circulating IGF-1 concentrations are largely beneficial during postnatal development, and closely associated with neurogenesis, somatic growth, reproduction, and wound-healing (Kenyon 2005; Yuan *et al.* 2012; Bartke *et al.* 2013). However, high concentrations during early postnatal development are also linked with reduced later-life survival relative to animals with lower circulating IGF-1 early in life, suggesting both the possibility of a trade-off and the importance of this particular period of development (Sonntag *et al.* 1999; Yuan *et al.* 2009, 2012; Panici *et al.* 2010; Svensson *et al.* 2011; Junnila *et al.* 2013).

IGF-1 has been hypothesized to mediate variation in life-history partitioning among wild mammalian species (Swanson & Dantzer 2014), but its role in life-history variation within such species remains largely unknown (Dantzer & Swanson 2012). Work with free-living populations

should allow us to elucidate the fitness consequences of naturally occurring variation in early IGF-1 concentrations, enhance our ability to identify potential targets of selection, and permit identification of sensitive windows during which endocrine markers may predict later-life traits. Here, we tested predictions of the hypothesis that juvenile IGF-1 concentrations mediate life-history trade-offs by using 26 years of detailed life-history data from wild female spotted hyenas (*Crocuta crocuta*) in Kenya. If IGF-1 mediates life-history trade-offs within species, then we would expect high juvenile IGF-1 concentrations to correlate positively with early benefits such as large juvenile body size, early age at first parturition, and enhanced survival to reproductive maturity, but also with the later-life cost of reduced lifespan after reproductive maturity (Dantzer & Swanson 2012). IGF-1 might influence life histories either directly or indirectly via intermediate effects on juvenile body size (Metcalfe & Monaghan 2003; Dmitriew 2011; Swanson & Dantzer 2014), so we also explored the independent effect of juvenile size on life-history traits. Finally, we explored the fitness consequences of contrasting life-history trajectories, focusing on annual reproductive success and the total number of cubs weaned by each female during her lifespan. If high juvenile concentrations of IGF-1 are consistent with a faster pace of life, then we would expect individuals exhibiting high concentrations early in life to have high annual reproductive success, but lower total reproductive success due to shortened lifespan. We investigated both direct and indirect influences of IGF-1 on reproductive fitness traits (Metcalfe & Monaghan 2003; Dmitriew 2011; Swanson & Dantzer 2014). Here indirect effects would be those mediated by IGF-1 effects on body size, and direct effects would be IGF-1 effects that occur independently of body size.

Materials and Methods

We measured plasma IGF-1 concentrations at 5.5-12.1 months of age in 70 juvenile female hyenas, 57 of which were followed until death, and inquired whether these juvenile IGF-1 concentrations predicted later trade-offs in the life histories of sampled individuals. We used 5.5 months as the lower limit of age range because this is the youngest age at which we can safely

immobilize juvenile spotted hyenas. We sought to limit variation in juvenile food acquisition by only looking at the period of maternal provisioning via lactation, so we used an upper age limit of 12.1 months, which is just before the average age of weaning in our study population (13.5 months, Holekamp *et al.* 1996). Social dominance plays a critical role in hyena society, and it can have profound, long-lasting direct and indirect effects on offspring fitness (Holekamp *et al.* 1996; Hofer & East 2003; Watts *et al.* 2009; Swanson, Dworkin & Holekamp 2011). Therefore, we included maternal dominance rank as a covariate in all our analyses. Furthermore, available food resources can directly influence the timing of first parturition in this species (Holekamp *et al.* 1996) so we also included local prey density as a covariate in our statistical models.

Relevant biology of spotted hyenas

Spotted hyenas are large-bodied carnivores with protracted early development; they do not reach full size until 30-36 months of age (Swanson *et al.* 2013). They live in groups, called clans, structured by linear dominance hierarchies (Kruuk 1972; Tilson & Hamilton 1984; Frank 1986). An individual's social dominance rank determines its priority of access to food at kills (Kruuk 1972; Tilson & Hamilton 1984; Frank 1986), and has profound fitness consequences (Holekamp *et al.* 1996; Hofer & East 2003; Watts *et al.* 2009). Here dominance rank matrices were constructed based on outcomes of dyadic agonistic interactions (Tilson & Hamilton 1984; Frank 1986). We standardized dominance rank to account for variation in clan size at time of immobilization; the highest-ranking animal in the clan has a relative rank of 1, while the lowest-ranking individual has a relative rank of -1. Because young hyenas learn their dominance ranks from their mothers and other group mates, but do not assume their proper positions in the clan's hierarchy until they are approximately 18 months of age (Holekamp & Smale 1993; Smale *et al.* 1993; Engh *et al.* 2000), we assigned each juvenile hyena its mother's dominance rank at the time of immobilization.

Spotted hyenas typically give birth to small litters containing only one or two cubs, which they nurse for up to 24 months. Female hyenas are philopatric and remain in the natal clan

throughout their lives, whereas virtually all males disperse after puberty (Smale, Nunes & Holekamp 1997; Höner *et al.* 2010; Davidian *et al.* 2016). Age at first parturition in our study population is generally between three and five years of age (Holekamp *et al.* 1996) so we used three years as the age of reproductive maturity in our survival analyses. Female spotted hyenas can live at least 24 years in the wild; our oldest female currently alive was born on October 7, 1992. For determination of life history-traits in female spotted hyenas, see electronic supplementary material.

Study site, data collection, and determination of life-history and fitness traits

Our subjects were part of a long-term field study of spotted hyenas within the Masai Mara National Reserve, Kenya. Behavioral data, demographic data, body size data and blood samples were collected between May 1988 and January 2015 from members of the Talek clan. Three samples with their associated data came from the Mara River clan, which has been observed since 2001. Ages of hyenas in their natal clans are known to ± 7 days based on methods described earlier (Holekamp *et al.* 1996). Juveniles in this study ranged from 5.5 to 12.1 mo. old (mean = 9.3 mo.) at the time of immobilization and blood sample collection. Hyenas younger than 5.5 months old could not be safely immobilized. As part of the ongoing study, juvenile hyenas are routinely anaesthetized with Telazol (Zoetis, South Bend, IN; 6.5 mg/kg) administered in a plastic dart fired from a CO_2-powered rifle (Telinject Inc., Saugus, CA). All immobilizations were conducted early in the morning, and all were carried out according to guidelines specified by the American Society of Mammalogists and approved by the Institutional Animal Care and Use Committee at Michigan State University (MSU; AUF #05/14-087-00). Following Telazol administration, hyenas were weighed and measured for four cranial and nine post-cranial linear morphological measurements (Swanson *et al.* 2011) (see also Figure B.1). Blood samples were taken from the jugular vein in heparinized vacutainer tubes, centrifuged, and plasma was drawn off, aliquoted, then promptly frozen in liquid nitrogen until it could be shipped on dry ice to MSU, where samples were stored at -80°C. The 5.5-12.1 mo. age interval

during which we sampled young female hyenas represents a phase of rapid growth for both size traits measured here, mass and shoulder height. However, the ages differ at which these traits reach maturity, defined as the age in months at which the predicted size for the trait equals 95% of the asymptotic value, or 'adult size.' Shoulder height reaches maturity at 21.03 mo. of age, whereas mass reaches maturity at 45.28 mo. of age (Swanson *et al.* 2013).

We calculated average local prey density as the mean number of ungulates counted within 100 meters of multiple 4-km transect lines run through each clan's territory at biweekly intervals during the six months prior to collection of each blood sample (Tables B.2-B.3) or during the six months prior to the birth of a female's first litter (Table B.4).

We determined age at first parturition was based on the initial tearing of the female's pseudopenis, indicated by the presentation of pink scar tissue on its posterior surface (Frank & Glickman 1994). This tearing reliably indicates that first parturition has occurred regardless of whether or not the first litter born is ever seen by observers when it appears above ground.

Female hyenas breed throughout their lives so we measured reproductive success only for females who survived past three years of age and had known death dates. We used the number of cubs a female weaned divided by the number of years between her third birthday and the birth of her last litter as a female's average annual reproductive success. We used the total number of cubs a female weaned during her lifetime as her overall reproductive success.

Because female spotted hyenas are philopatric, and because we were observing our study clans daily, we recorded each female's date last seen as her date of death. With six exceptions, we included samples from females disappearing before January 2014 to allow ample opportunity for reappearance. The six exceptions were females still alive at the end of the study period.

Of the 35 females that survived to or past three years of age and had known death dates, five females failed to bear any offspring and four females failed to raise offspring to weaning so were not included in the analysis of annual reproductive success (Table B.7; Figure

B.3). All 35 females were included in the analysis of number of cubs weaned (Table B.8; Figure B.4), including the five females that did not give birth to any offspring and four other females that failed to wean any offspring. We performed model comparison using Akaike's Information Criteria corrected for small sample sizes (AICc) (Burnham & Anderson 2002). Models are comparable if ΔAICc<2.0.

Measuring IGF-1 concentrations in plasma

We measured IGF-1 concentrations in plasma collected from hyenas immobilized between 1990 and 2014. To quantify plasma IGF-1 levels, we used a commercially available enzyme immunoassay kit (EIA; ALPCO Diagnostics, Salem, NH). We validated this assay for spotted hyenas using the 'spike and recovery' method following kit protocol. Briefly, 25µl of kit sample buffer (0 ng/ml IGF-1) were added to 25µl plasma aliquots from three different individuals. The measured IGF-1 concentration served as the baseline concentration. Separate 25µl plasma aliquots from the same three hyena plasma samples were then spiked with 25µl of 50 ng/ml IGF-1 (kit standard E) and measured for IGF-1 concentrations. The measured IGF-1 concentration served as the observed value. The expected value for each hyena sample was calculated as the sample's baseline concentration plus the spiked concentration of 50 ng/ml. Percent recovery was calculated as the [observed value/expected value x 100] (Table B.1). Our average percent recovery of 116.1% fell within the kit's acceptable range of 70-130%. Hyena plasma samples were diluted to 1:20 so measured concentrations fell within the kit standard curve (Figure B.2). All samples were run in duplicate and randomly assigned to plates (n = 11 plates). Mean intra- and inter-assay coefficients of variation for the IGF-1 immunoassays were 3.06% and 10.5%, respectively.

Plasma samples were kept frozen at -80°C, but the length of time frozen varied between 3 months and 24 years. To assess whether freezer storage time affected IGF-1 concentrations, we used a paired t-test to compare variation between randomly chosen subsets of plasma samples frozen before and after 1999 (n = 30, each). We found no significant difference

between the two subsets (t_{58} = -0.72, P = 0.47), suggesting that storage time was not a confounding variable. Additionally, a simple linear regression revealed no influence of freezer storage time on IGF-1 concentrations (r^2 = 0.007, t_{68} = 0.69, P =0.49).

Statistical analyses

Due to the opportunistic nature of our sampling methods, we could only sample juveniles once so there were no repeated measures in any of our analyses. We first used multiple linear regression to explore the independent contributions of maternal dominance rank, age measured in months, and local prey density as predictors of juvenile concentrations of IGF-1. We calculated age (in months) using known birthdates, and we calculated prey density as the average local prey density during the 6 months prior to immobilization. Residuals of this regression model were normally distributed (Shapiro-Wilk W = 0.98, P = 0.32).

We then used multiple linear regression to assess the influences of juvenile IGF-1, maternal dominance rank at the time of juvenile immobilization, and local prey density on relative juvenile size at the time of sampling (Table B.3). We included all three predictor variables in a single model because there was no evidence of multi-collinearity among them, and we believed them all to be biologically relevant to predicting juvenile size. We used residuals from two measures of relative juvenile body size at age of blood sampling: body mass and shoulder height (shown as measure “c” in Figure B.1). An individual that was ‘large for its age’ had a positive deviation (residual), and an individual ‘small for its age’ had a negative deviation (Altmann & Alberts 2005). One individual had neither mass nor shoulder height recorded so was not included in models with size; however, this individual did have an IGF-1 measurement, so was included in all other relevant models. Two other individuals had no shoulder height recorded but did have IGF-1 measurements so they were included in relevant models excluding size. Residuals for all the models compared were normally distributed (Shapiro-Wilk $W \geq 0.96$, $P \geq 0.16$).

To assess how age at first parturition varied among females, we used multiple linear regression with juvenile concentrations of IGF-1, body mass residuals, maternal dominance rank, and local prey density as continuous predictor variables. Maternal dominance rank equaled the rank of the subject's mother at the time of its immobilization as a juvenile, and local prey density equaled the density during the 6 months prior to the date of a female's first parturition. We included these two variables in both models. We were interested in comparing direct and indirect effects of IGF-1 on age at first parturition, so we included IGF-1 and body mass residuals in separate models. We assessed relative model fit using Akaike's Information Criteria corrected for small sample sizes (AICc). Models are comparable if ΔAICc<2.0. For model comparison, we included only individuals with both IGF-1 and body mass. Model residuals did not differ significantly from a normal distribution (Shapiro-Wilk $W \geq 0.95$, $P \geq 0.18$).

To analyze the contributions of IGF-1 to survival to and beyond reproductive maturity, we performed Cox proportional-hazards regression analyses using the 'coxph' function in the 'survival' package in R (Therneau 2014). We opted to use proportional hazards to model survival because we did not expect IGF-1 concentrations to have a linear relationship with lifespan. A negative hazard rate coefficient (β) indicates a decreased probability of survival with increasing value of the predictor variable; a positive hazard rate coefficient (β) indicates an increased probability of survival with increasing value of the predictor variable. A hazard ratio ($exp(\beta)$) equal to 2, for example, indicates that individuals are twice as likely to survive with increasing values of the predictor variable. We included six individuals still alive in January 2014 as right-censored data points. Because we were specifically interested in both direct and indirect (mediated via body size) influences of IGF-1 on survival, we limited our comparison to that between two models: one with IGF-1 and one with body mass. We included maternal dominance rank as a covariate in both analyses due to previous findings on survival in this population (Watts *et al.* 2009). Visual inspection of scaled Schoenfeld residuals showed no violation of the assumption of proportional hazards, influential observations, or nonlinearity.

Although both IGF-1 and body size measures were modeled as continuous variables in all statistical analyses, we divided these predictors into tertiles for illustrative purposes in some of our figures.

We used multiple linear regression to investigate two different aspects of reproductive fitness: annual reproductive success and lifetime reproductive success. To predict average annual reproductive success, we compared three multiple linear regression models with maternal dominance rank and either IGF-1, age at first parturition or juvenile body mass as a covariate. In these models, we only included females with IGF-1 values, juvenile body mass measurements, and at least one litter (n = 29). To predict lifetime reproductive success, we compared four multiple linear regression models with maternal dominance rank and either IGF-1, juvenile body mass, age at first parturition, or lifespan as a covariate. We assessed relative model fit using Akaike's Information Criteria corrected for small sample sizes (AICc). Models are comparable if ΔAICc<2.0.

For all model analyses, we centered all continuous predictor variables with a mean of 0 and standard deviation of 1. Model residuals did not differ significantly from a normal distribution (Shapiro-Wilk $W \geq 0.94$, $P \geq 0.08$), and there was no evidence of multicollinearity (square root of variance inflation factor < 2). For models of lifetime reproductive success, we performed a square-root transformation of the dependent variable (total number of cubs weaned) in order to meet assumptions of normality (value of 0.5 added to counts of 0 cubs weaned). Following the transformation, model residuals did not differ significantly from a normal distribution (Shapiro-Wilk $W \geq 0.95$, $P \geq 0.18$).

All statistical analyses were performed in R v. 3.2.3 (R Development Core Team 2014).

Results

Predictors of juvenile IGF-1 concentrations

None of our model variables significantly predicted concentrations of IGF-1 in juvenile females sampled between 5.5 and 12.1 months of age (maternal dominance rank: t_{65} = 0.28, P = 0.782; age: t_{65} = -0.29, P = 0.770; prey density: t_{65} = -0.30, P = 0.768; Table B.2).

Juvenile body size

Female hyenas with higher juvenile IGF-1 concentrations were heavier, but not taller, for their age than females with lower juvenile concentrations (mass: t_{64} = 3.80, P = 0.0003; Figure 3.1A; shoulder height: t_{62} = 1.28, P = 0.21; Table B.3). Juveniles born to high-ranking mothers were both heavier and taller than those born to low-ranking mothers (mass: t_{64} = 2.86, P = 0.006; shoulder height: t_{62} = 2.06, P = 0.04; Table B.3). Average local prey density during the 6 months before blood collection did not influence either measure of juvenile size (Table B.3).

Age at first parturition

Among wild female hyenas, we found that higher juvenile IGF-1 concentrations predicted earlier age at first parturition (t_{34} = -2.15; P = 0.039; Figure 3.1B) after accounting for maternal rank and average local prey density (Table B.4). This model fit the data better than a model containing mass instead of IGF-1 (Table B.4a); mass did not significantly predict age at first parturition (Table 3.4b).

Survival to reproductive maturity

Females that were heavier as juveniles survived proportionally better than did lighter females (z = -2.48, P = 0.013; Figure 3.2; Table B.5). Maternal rank did not significantly predict survival to reproductive maturity. This model fit the data better than a model containing IGF-1 instead of mass (Table B.5).

Survival after reproductive maturity

Female hyenas with higher juvenile IGF-1 concentrations had shorter life expectancies after reaching reproductive maturity than did females with lower IGF-1 concentrations in early

life (z = -1.28, P = 0.023; Figure 3.3; Table B.6). Daughters of high-ranking mothers also tended to survive better than did daughters of low-ranking mothers (z = -1.95, P = 0.052; Table B.6). Juvenile mass did not predict survival in adulthood and was not included in the best-supported model.

Fitness measures

Females with younger ages at first parturition experienced higher annual reproductive success than did females that started breeding when they were older (t_{25} = -2.82, P = 0.009; Tables B.7a & B.7b; Figure B.3). Longer life expectancy after reaching reproductive maturity and higher maternal dominance rank significantly predicted larger numbers of weaned cubs (t_{26} = 7.55, P <0.0001; Tables B.8a & B.8b; Figure B.4).

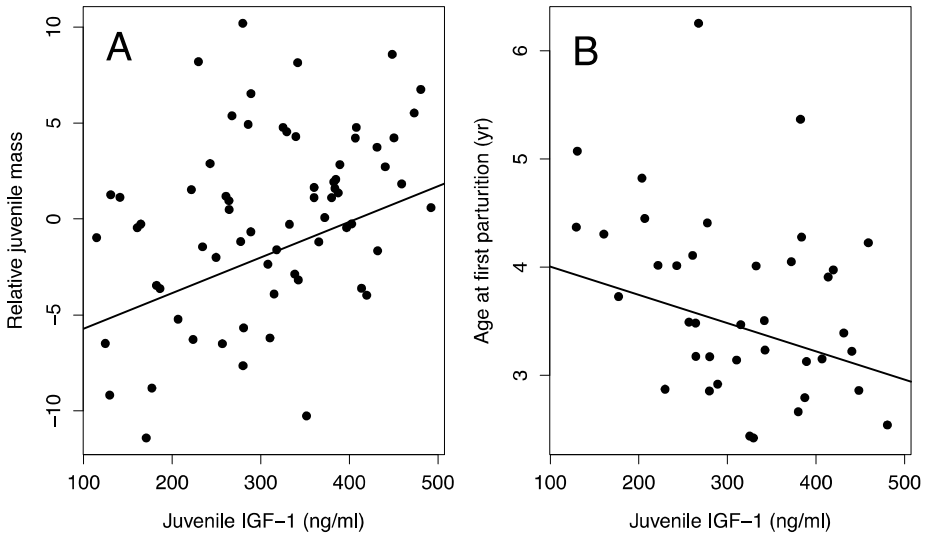

Figure 3.1. Female hyenas exhibiting higher concentrations of IGF-1 as juveniles were (A) heavier as juveniles and (B) gave birth to their first litter at younger ages. Females with higher juvenile plasma IGF-1 concentrations were (A) relatively heavy for their age (t_{64} = 3.80, P = 0.0006) and (B) gave birth to their first litters at younger ages (t_{34} = -2.15; P = 0.039) than did those with lower juvenile plasma IGF-1 concentrations. Regression lines are from the linear regression models.

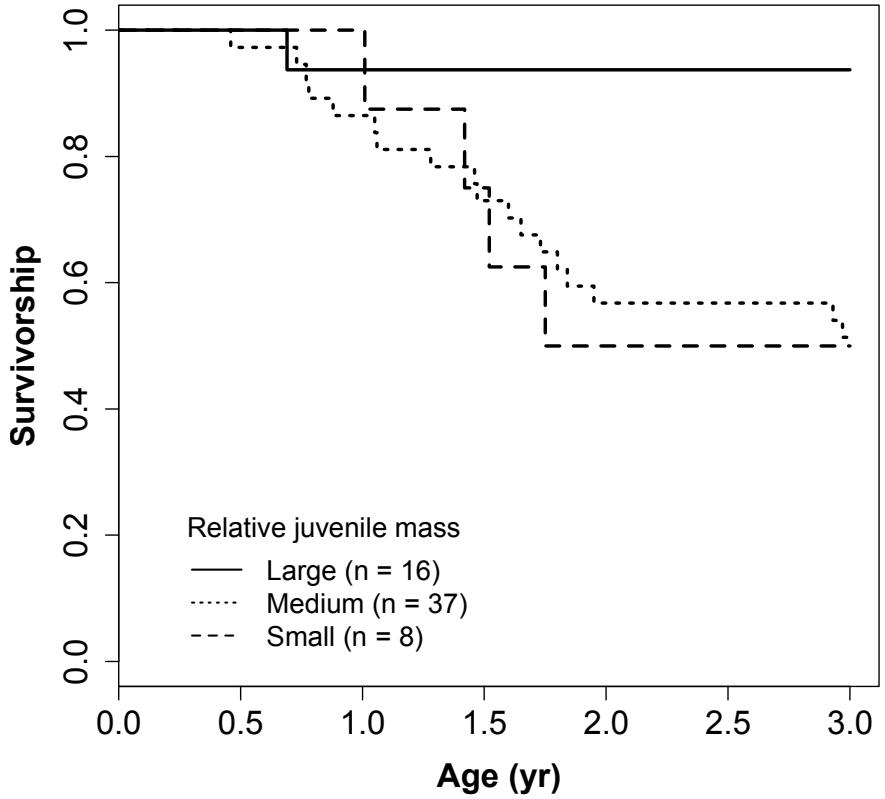

Figure 3.2. Survival curves to reproductive maturity for juvenile females of varying relative mass. Female hyenas that were relatively heavy as juveniles survived to reproductive maturity at higher rates than those that were relatively light as juveniles (likelihood ratio test: n= 61, $\chi^2 = 6.17$, df = 1, $P = 0.01$). Right-censored tick marks (+) indicate females that survived past 3 years of age (n = 39). Relative mass was statistically analyzed as a continuous variable (Table B.5), but partitioned into tertiles for illustrative purposes only.

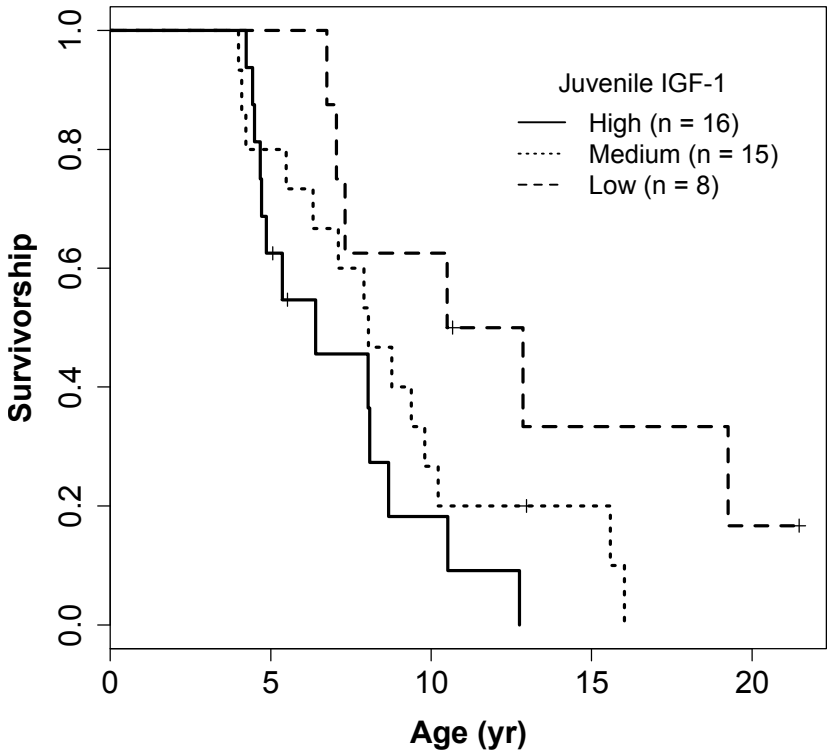

Figure 3.3. Survival curves after reproductive maturity for females exhibiting varying concentrations of IGF-1 as juveniles. Female hyenas exhibiting higher concentrations of IGF-1 as juveniles experienced significantly lower rates of survival after reproductive maturity than did hyenas exhibiting lower juvenile IGF-1 concentrations (likelihood ratio test: n= 39, χ^2 = 7.88, df = 2, *P* = 0.02). Tick marks (+) indicate females alive at the end of the study period (n = 6). IGF-1 was statistically analyzed as a continuous variable (Table B.6), but classified into tertiles for illustrative purposes only.

Discussion

IGF-1 concentrations decrease with age over the lifespan in spotted hyenas (unpublished data) as they do in other species (Bartke *et al.* 2013), but age did not influence

concentrations among our study subjects between 5.5 and 12.1 months of age (t_{65} = -0.29, P = 0.77; Table B.2). Social dominance rank has been shown to predict IGF-1 concentrations in adult baboons (Sapolsky & Spencer 1997), but the juvenile hyenas sampled here were still in the early process of learning their social ranks. We found no influence of maternal dominance rank on juvenile IGF-1 concentrations (t_{65} = 0.28, P = 0.78; Table B.2), although maternal dominance rank did positively influence juvenile size (Figure 3.1A; Table B.3). This suggests that social rank acts independently of IGF-1 in mediating size; this has also been found in juvenile mandrills (Bernstein *et al.* 2012). Lastly, there appeared to be no effect of prey density on IGF-1 concentrations (t_{65} = -0.30, P = 0.77; Table B.2).

Postnatal body size is a crucial life-history trait that figures prominently in hallmark examples of life-history trade-offs (Metcalfe & Monaghan 2003; Dmitriew 2011). Large body size appears to be one of the first traits that young animals can exploit to optimize their interactions with their environment and thus survive to reproductive maturity. If IGF-1 indeed mediates the life-history trade-offs associated with the pace of life continuum, then it should have important effects on growth (Miller *et al.* 2002; Dantzer & Swanson 2012). Hyenas undergo much rapid growth before one year of age (Swanson *et al.* 2013), and our results suggest that IGF-1 concentrations may be promoting variable gains in body size during this period (Figure 3.1A; Table B.3). Local autocrine or paracrine secretion of IGF-1 and other components of the somatotropic axis, such as growth hormone and various IGF-1 binding proteins, are also likely to affect growth (Hossner, McCusker & Dodson 1997; Yakar *et al.* 1999; Kappeler *et al.* 2009), but these remain unexamined in spotted hyenas.

In laboratory and domesticated mammals, IGF-1 helps stimulate gonadal hormone production and thus plays a role in mediating the timing of reproductive maturity (Hiney *et al.* 1996; Taylor *et al.* 2008; Sparkman, Byars & Ford 2010; Yuan *et al.* 2012; Bartke *et al.* 2013). Here we found that higher juvenile concentrations of IGF-1 predicted earlier ages at first parturition among female hyenas (Figure 3.1B, Table B.4b). IGF-1 predicted age at first

parturition better than did relative juvenile mass, suggesting that reproduction may be more tightly regulated by internal metabolic signals than by overall body size. Female spotted hyenas of all social ranks breed year-round, and do not monopolize mating opportunities, so there is no competitive advantage to breeding at a younger age. Nevertheless, females that give birth to their first litter earlier in life appear to benefit from higher annual reproductive success than females that start breeding later in life (Figure B.3). This may give the former a competitive advantage, and is also characteristic of a 'fast' pace of life (Promislow & Harvey 1990; Stearns 1992; Descamps *et al.* 2006).

Given the importance of reproductive success to fitness, theory predicts that survival to reproductive maturity should be under strong selection, with selection for survival weakening as individuals age after reproductive maturity (Williams 1957; Hamilton 1966). Consistent with predictions of our hypothesis, female hyenas with high juvenile IGF-1 appeared to experience a trade-off later in life, and had shorter life expectancies after reproductive maturity than did females with low juvenile IGF-1 values (Figure 3.3, Table B.6). Juvenile mass did not predict survival in adulthood, suggesting that IGF-1 might be operating independently of mass in relation to this life-history trait. Spotted hyenas breed throughout their lives, so reduced survival after puberty was associated with fewer reproductive opportunities and therefore lower lifetime reproductive success (Figure B.1, Table B.8). However, it is possible that those females expressing higher juvenile IGF-1 concentrations, and having earlier ages at first parturition, offset their shorter lifespans by increasing annual reproductive success (Figure B.3, Table B.8a).

The pleiotropic effects of IGF-1 appeared to restrict hyenas' ability to enjoy high survival both before and after reproductive maturity, which is consistent with the idea that senescence begins at the onset of reproductive maturity (Williams 1957; Hamilton 1966). Despite living in a variable environment with fluctuating food abundance and natural predators, our subjects experienced survivorship rates similar to those seen in laboratory rodents (Sonntag *et al.* 1999;

Panici *et al.* 2010; Svensson *et al.* 2011; Yuan *et al.* 2012; Junnila *et al.* 2013; Bartke *et al.* 2013) and expected by theory (Williams 1957; Stearns 1992). The accumulation of cellular damage has been proposed as one cause of death in animals (Flatt & Heyland 2011; Dantzer & Swanson 2012), but lions (*Panthera leo*) and humans represent the largest sources of mortality for spotted hyenas (Kruuk 1972; Watts & Holekamp 2009), so most die violent deaths. To explain the relatively poor survival after reproductive maturity of hyenas with high juvenile IGF-1, we hypothesize that individuals with higher juvenile IGF-1 concentrations might engage in more risky behaviors, which are characteristic of a high energy metabolism (Svensson *et al.* 2005; Stamps 2007; Biro & Stamps 2010; Baldini *et al.* 2013). However, this idea remains to be assessed.

We were unable to document within-subject variation in IGF-1 concentrations among our subjects because we could not repeatedly immobilize young hyenas. Although it would have been interesting to determine how stable IGF-1 concentrations are in these animals, we find it encouraging that clear patterns of significance emerged with only one sample per individual. Furthermore, in contrast to other hormones, which may be highly labile, relative circulating IGF-1 concentrations vary consistently over time and show high intra-individual repeatability in other species (Roberts *et al.* 1990; Obese *et al.* 2008; Yuan *et al.* 2009; Kappeler *et al.* 2009). Lastly, within-individual IGF-1 concentrations at 6, 12, and 18 months all correlated with survival in developing mice (only the correlation at 6 months was significant; Yuan et al. 2009), so our results are not without precedent.

The initial benefits and delayed costs associated with high juvenile concentrations of IGF-1 in spotted hyenas are consistent with life-history theory, suggesting that IGF-1 may mediate life history trade-offs within, as well as between, species in the wild. Our results support the hypothesis that IGF-1 functions to optimize life history variation, and suggest that circulating IGF-1 concentrations measured during early postnatal development in wild mammals can be used to predict important adult traits not expressed until many years later. Our work elucidates

the fitness correlates of naturally occurring variation in early IGF-1 concentrations, and thus sheds considerable new light on mechanisms mediating life-history trade-offs in a fashion that would be impossible in controlled laboratory settings.

APPENDIX

Supplementary Material for Chapter 3

Table B.1. A commercially available enzyme immunoassay kit was validated for spotted hyenas using the 'spike and recovery' method following kit protocol.

	Baseline	Recovered	Expected	% Recovery
Sample 1	229.44	301.72	254.44	118.58%
Sample 2	179.57	252.24	204.57	123.30
Sample 3	267.91	311.74	292.91	106.43

Results from the spike and recovery method for assay validation. Average percent recovery was 116.1% ((recovered value/expected value) x 100), with 70-130% as the acceptable range per kit instructions.

Table B.2. Concentrations of juvenile IGF-1 concentrations in sampled female hyenas were not significantly influenced by maternal dominance rank, age at sampling, or prey density. Significant effects are highlighted in boldface at a significance level of $\alpha = 0.05$.

Trait	Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Juvenile IGF-1	**Intercept**	**305.31 ± 12.22**	**280.90 − 329.71**	**24.24**	**65**	**<0.0001**
	Mat. dom. rank	6.49 ± 23.35	-40.15 − 53.13	0.28	65	0.782
	Age	-2.33 ± 7.92	-18.15 − 13.49	-0.29	65	0.770
	Prey density	-0.06 ± 0.19	-0.43 − 0.322	-0.30	65	0.768

Results from a multiple linear regression model predicting concentrations of IGF-1 among 69 juvenile female spotted hyenas sampled between 5.5 and 12.1 months of age. All predictor variables were centered with a mean of 0 and standard deviation of 1. Hyenas were assigned their mother's dominance rank (Mat. dom. rank) at the time of immobilization; a relative rank of 1 was the highest-ranking animal in the clan, and the individual with a relative rank of -1 was the lowest-ranking. Age at sampling was calculated in months based on known birthdates. Prey

density was calculated as the average local prey density during the 6 months prior to immobilization.

Table B.3. Juveniles with high concentrations of IGF-1 were heavier, but not taller, than juveniles with low concentrations of IGF-1. Juveniles born to high-ranking mothers were both heavier and taller than those born to low-ranking mothers. Mean mass ± SE = 22.88 ± 0.74 kg; mean shoulder height ± SE = 60.39 ± 0.60 cm. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Trait	Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Juvenile mass	Intercept	-7.54 ± 1.90	-11.3 − 3.7	-3.96	65	**<0.001**
	Juvenile IGF-1	**0.02 ± 0.00**	**0.01 − 0.03**	**3.80**	**65**	**<0.001**
	Mat. dom. rank	**2.67 ± 0.93**	**0.82 − 4.53**	**2.89**	**65**	**0.005**
	Prey density	0.01 ± 0.01	-0.01 − 0.02	1.23	65	0.224
Juvenile shoulder height	Intercept	-1.59 ± 1.71	-5.0 − 1.83	-0.09	63	0.356
	Juvenile IGF-1	$6.1\times10^{-3} \pm 4.5\times10^{-3}$	-2.8×10^{-3} − 0.02	1.36	63	0.178
	Mat. dom. rank	**1.72 ± 0.84**	**0.05 − 3.40**	**2.06**	**63**	**0.043**
	Prey density	$-5.0\times10^{-3} \pm 5.0\times10^{-3}$	-0.18 − 0.01	-0.73	63	0.470

Results from two separate multiple linear regression models predicting mass among 69 juvenile female spotted hyenas and shoulder height among 67 juvenile female spotted hyenas. Juvenile mass and juvenile shoulder height were expressed as residuals from separate linear regressions predicting body mass and shoulder height by age, respectively. An individual that was ‘large for its age’ would have a positive deviation (residual) from this line, and an individual ‘small for its age’ would have a negative deviation. Shoulder height data were unavailable for two individuals. For both analyses shown in Table 3.3, ‘prey density’ was calculated as the average local prey density during the 6 months prior to immobilization and blood sampling.

Table B.4a. Comparison of two multiple linear regression models predicting age at first parturition using Akaike's Information Criteria corrected for small sample sizes (AICc). Models are comparable if ∆AICc<2.0. Only individuals with both IGF-1 and mass measurements were included in models for comparison (n = 37).

	df	**logLik**	**AICc**	**∆AICc**	**Weight**
Model 1	5	-35.30	82.5	0.0	0.85
Model 2	5	-37.04	86.0	3.5	0.15

Table B.4b. Female hyenas were significantly younger at their first parturition when they exhibited higher juvenile concentrations of IGF-1, were born to higher-ranking mothers, and experienced higher local prey density during the 6 months before parturition than other females. Mean age at first parturition among our samples ± SE = 3.67 ± 0.13 yrs. Models correspond to those in Table 3.4a except that all available data points were used in Model 1 (1 individual with IGF-1 but no juvenile mass measurement). Significant effects are highlighted in boldface at a significance level of α = 0.05.

Model 1: n = 38, adjusted R^2 = 0.36, p = 0.0004

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**3.67 ± 0.11**	**3.45 − 3.90**	**33.11**	**34**	**<0.0001**
Juvenile IGF-1	**$-2.6 \times 10^{-3} \pm 1.2 \times 10^{-3}$**	**-5.1×10^{-3} − -0.1×10^{-3}**	**-2.15**	**34**	**0.039**
Mat. dom. rank	**-0.57 ± 0.21**	**-1.00 − -0.15**	**-2.75**	**34**	**0.009**
Prey density	**$-2.7 \times 10^{-3} \pm 1.1 \times 10^{-3}$**	**-0.4×10^{-3} − 5.0×10^{-3}**	**2.43**	**34**	**0.021**

Model 2: n =37, adjusted R^2 = 0.32, p = 0.001

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**$3.642 \pm 1.1 \times 10^{-1}$**	**3.41 − 3.88**	**31.77**	**33**	**<0.0001**
Juvenile mass	$-2.9 \times 10^{-5} \pm 2.7 \times 10^{-2}$	-0.06 − 0.06	-1.0×10^{-3}	33	0.999
Mat. dom. rank	**$-0.68 \times 10^{-1} \pm 0.25$**	**-1.18 − -0.18**	**-2.75**	**33**	**0.010**
Prey density	**$-2.9 \times 10^{-3} \pm 1.2 \times 10^{-3}$**	**-0.5×10^{-3} − 5.2×10^{-3}**	**2.46**	**33**	**0.019**

Results from two multiple linear regression models predicting age at first parturition, expressed in years. All predictor variables were centered with a mean of 0 and standard deviation of 1. Juvenile mass was expressed as residuals from a linear regressions predicting body mass by age. An individual that was 'large for its age' would have a positive deviation (residual) from this line, and an individual 'small for its age' would have a negative deviation. 'Prey density' for this analysis was calculated as the average local prey density during the 6 months prior to the date of first parturition.

Table B.5. Results from two separate Cox proportional hazards models predicting survival to reproductive maturity. Heavier juvenile female hyenas experienced significantly greater survival to reproductive maturity than did smaller females. Model 2 was the best-supported model based on likelihood ratio tests. One individual had no mass recorded so was not included in Model 2, in which mass was a predictor variable; however, this individual did have an IGF-1 value so was included in Model 1. Significant effects are highlighted in boldface at a significance level of $\alpha = 0.05$.

Model 1: n= 62; $\chi^2 = 3.29$; df = 2, $P = 0.19$, model log likelihood = -88.59

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile IGF-1	-0.32	0.72	-1.46	0.145
Mat. dom. rank	-0.40	0.67	-1.06	0.288

Model 2: n= 61; $\chi^2 = 6.20$; df = 2, $P = 0.45$, model log likelihood = -86.67

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile mass	**-0.11**	**0.90**	**-2.27**	**0.023**
Mat. dom. rank	-0.07	0.93	-0.17	0.868

All predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. A negative hazard rate coefficient (β) indicates a decreased probability of survival with increasing value of the predictor variable. A hazard ratio (calculated as *exp*(β)) of 2, for example, indicates that individuals are twice as likely to survive with increasing values of the predictor variable.

Table B.6. Results from two separate Cox proportional hazards models predicting survival past reproductive maturity. Individuals with higher juvenile concentrations of IGF-1 were more likely to survive than those with lower juvenile concentrations. Model 1 was the best-supported model based on likelihood ratio tests. One individual had no mass recorded so was not included in Model 2, in which mass was a predictor variable; however, this individual did have an IGF-1 value so was included in Model 1. Significant effects are highlighted in boldface at a significance level of $\alpha = 0.05$.

Model 1: n= 39; $\chi^2 = 7.88$; df = 2, $P = 0.02$, model log likelihood = -88.98

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
IGF-1	**0.45**	**1.57**	**2.28**	**0.023**
Mat. dom. rank	-0.67	0.51	-1.95	0.052

Model 2: n= 38; $\chi^2 = 2.98$; df = 2, $P = 0.23$, model log likelihood = -88.23

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile mass	0.02	1.02	0.61	0.539
Mat. dom. rank	-0.62	0.54	-1.79	0.074

All predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. A negative hazard rate coefficient (β) indicates a decreased probability of survival with increasing value of the predictor variable. A positive hazard rate coefficient (β) indicates an increased probability of survival with increasing value of the predictor variable. A hazard ratio (calculated as *exp*(β)) of 2, for example, indicates that individuals are twice as likely to survive with increasing values of the predictor variable.

Table B.7a. Comparison of three multiple linear regression models predicting annual reproductive success using Akaike's Information Criteria corrected for small sample sizes (AICc). Models are comparable if ∆AICc<2.0. Females were only included in models for comparison if they survived past 3 years of age, had known death dates, had successfully weaned at least one offspring, and had complete IGF-1, mass, and age at first parturition measurements (n = 25).

	df	**logLik**	**AICc**	**∆AICc**	**Weight**
Model 1	4	8.17	-6.3	0.0	1
Model 2	4	0.52	9.0	15.3	<0.001
Model 3	4	-0.24	10.5	16.8	<0.001

Table B.7b. Results from three linear models to determine a female's annual reproductive success. Models correspond to those in Table 3.7a except that all available data points for each model were used below. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Model 1: n =26, adjusted R^2 = 0.54, p = 0.00005

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**0.65 ± 0.04**	**0.57 − 0.73**	**17.43**	**23**	**<0.0001**
Age at first parturition	**-0.20 ± 0.05**	**-0.30− -0.11**	**-4.34**	**23**	**0.0002**
Mat. dom. rank	0.06 ± 0.08	-0.11 − 0.22	0.71	23	0.486

Model 2: n =26, adjusted R^2 = 0.22, p = 0.022

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**0.65 ± 0.05**	**0.55 − 0.75**	**13.38**	**23**	**<0.0001**
Juvenile IGF-1	$6.9 \times 10^{-4} \pm 5.3 \times 10^{-4}$	$-4.1 \times 10^{-4} - 1.8 \times 10^{-3}$	1.30	23	0.207
Mat. dom. rank	**0.23 ± 0.09**	**0.05 − 0.42**	**2.57**	**23**	**0.017**

Model 3: n =25, adjusted R^2 = 0.17, p = 0.048

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**0.65 ± 0.05**	**0.54 − 0.76**	**12.48**	**23**	**<0.0001**
Juvenile mass	0.008 ± 0.01	-0.02− 0.04	0.58	23	0.571
Mat. dom. rank	**0.22 ± 0.10**	**0.02− 0.43**	**2.26**	**23**	**0.034**

A female's annual reproductive success was calculated as the number of her weaned cubs divided by her reproductive lifespan. Both female and male offspring are included.

Results from three multiple linear regression models predicting age at first parturition, expressed in years. All predictor variables were centered with a mean of 0 and standard deviation of 1.

Table B.8a. Comparison of four multiple linear regression models predicting lifetime reproductive success using Akaike's Information Criteria corrected for small sample sizes (AICc). Models are comparable if ∆AICc<2.0. Females were only included in models for comparison if they survived past 3 years of age, had known death dates, and had complete IGF-1, mass, and age at first parturition measurements (n = 29).

	df	**logLik**	**AICc**	**∆AICc**	**Weight**
Model 1	4	-19.73	49.1	0.0	1
Model 3	4	-31.75	73.2	24.0	<0.001
Model 2	4	-33.16	76.0	26.8	<0.001
Model 4	4	-34.07	77.8	28.7	<0.001

Table B.8b. Results from three linear models to determine a female's lifetime reproductive success. All available data points for each model were used below. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Model 1: n =35, adjusted R^2 = 0.78, p = 1.39×10^{-11}

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**2.96 ± 0.25**	**2.45 − 3.46**	**11.95**	**32**	**<0.0001**
Lifespan	**0.65 ± 0.07**	**0.51− 0.79**	**9.63**	**32**	**<0.0001**
Mat. dom. rank	**1.12 ± 0.47**	**0.16 − 2.08**	**2.39**	**32**	**0.023**

Model 2: n =32, adjusted R^2 = 0.19, p = 0.030

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**2.86 ± 0.46**	**1.93 − 3.79**	**6.28**	**29**	**<0.0001**
Juvenile mass	0.14 ± 0.12	-0.10 − 0.38	1.17	29	0.252
Mat. dom. rank	1.75 ± 0.89	-0.08 − 3.57	1.96	29	0.060

Model 3: n =34, adjusted R^2 = 0.19, p = 0.01

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**3.06 ± 0.48**	**2.08 − 4.05**	**6.33**	**31**	**<0.0001**
Juvenile IGF-1	$-5.5\times10^{-3} \pm 5.4\times10^{-3}$	$-0.16 - 5.4\times10^{-3}$	-1.03	31	0.310
Mat. dom. rank	**2.68 ± 0.89**	**0.87 − 4.50**	**3.01**	**31**	**0.005**

Model 4: n =30, adjusted R^2 = 0.09, p = 0.11

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	**3.26 ± 0.56**	**2.12 − 4.40**	**5.86**	**27**	**<0.0001**
Age at first parturition	-0.19 ± 0.69	-1.59 − 1.22	-0.27	27	0.788
Mat. dom. rank	2.01 ± 1.09	-0.22− 4.24	1.85	27	0.075

A female's annual reproductive success was calculated as the number of her weaned cubs divided by her reproductive lifespan. Both female and male offspring are included.
Results from two multiple linear regression models predicting age at first parturition, expressed in years. All predictor variables were centered with a mean of 0 and standard deviation of 1.

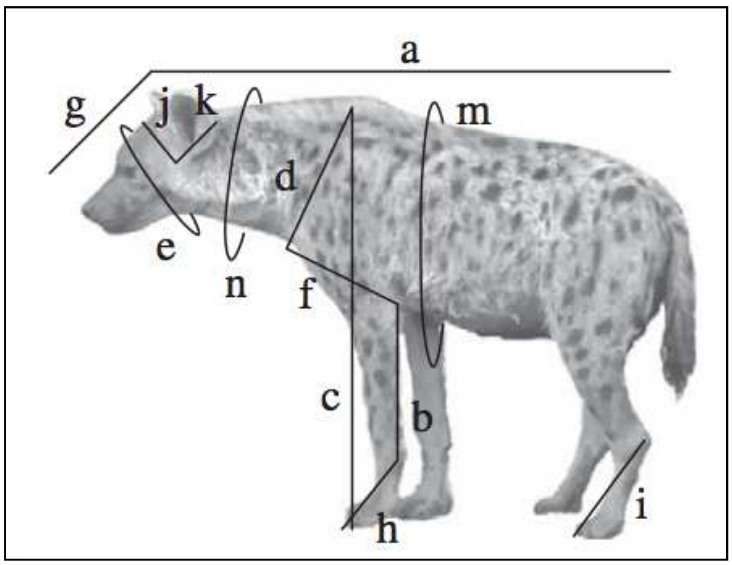

Figure B.1. Morphological traits measured during routine immobilization of study animals. Shoulder height used for this study indicated by measure “c” above. Reproduced from: Swanson, E.M., Dworkin, I. & Holekamp, K.E. (2011) Lifetime selection on a hypoallometric size trait in the spotted hyena. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 3277–85.

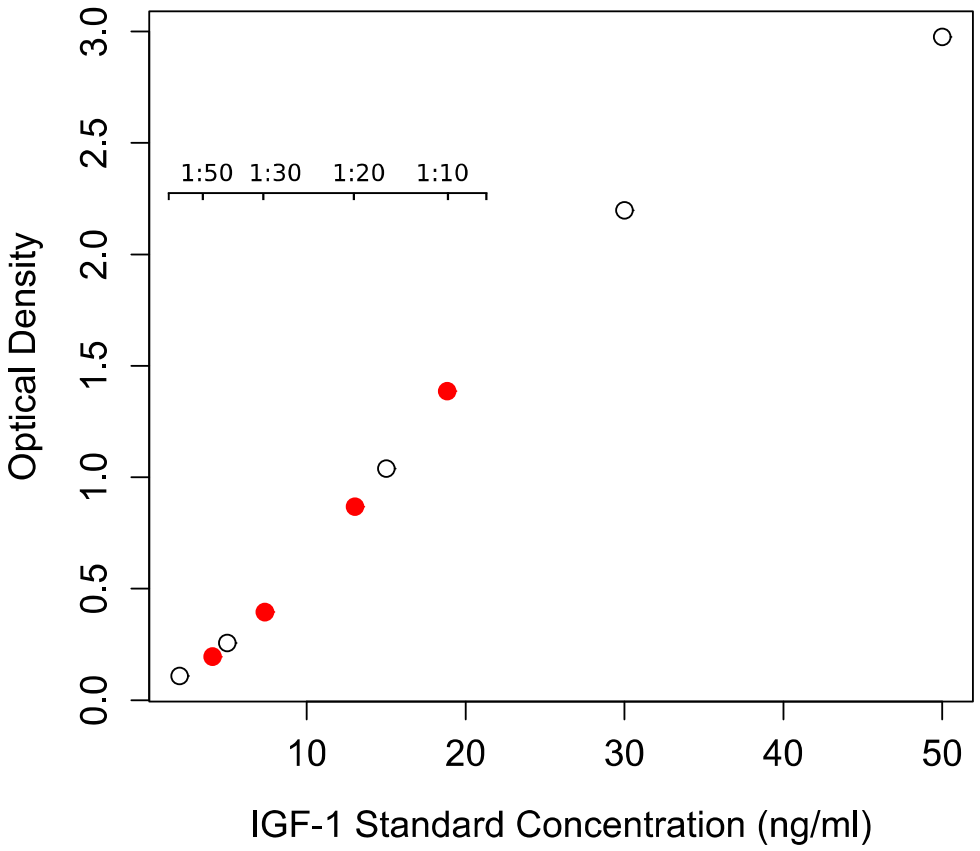

Figure B.2. IGF-1 standards (open circles; bottom x-axis) and serial dilutions of a pooled sample of hyena plasma (filled circles; inset x-axis) plotted against optical density values (y-axis). Samples for this study were diluted to 1:20.

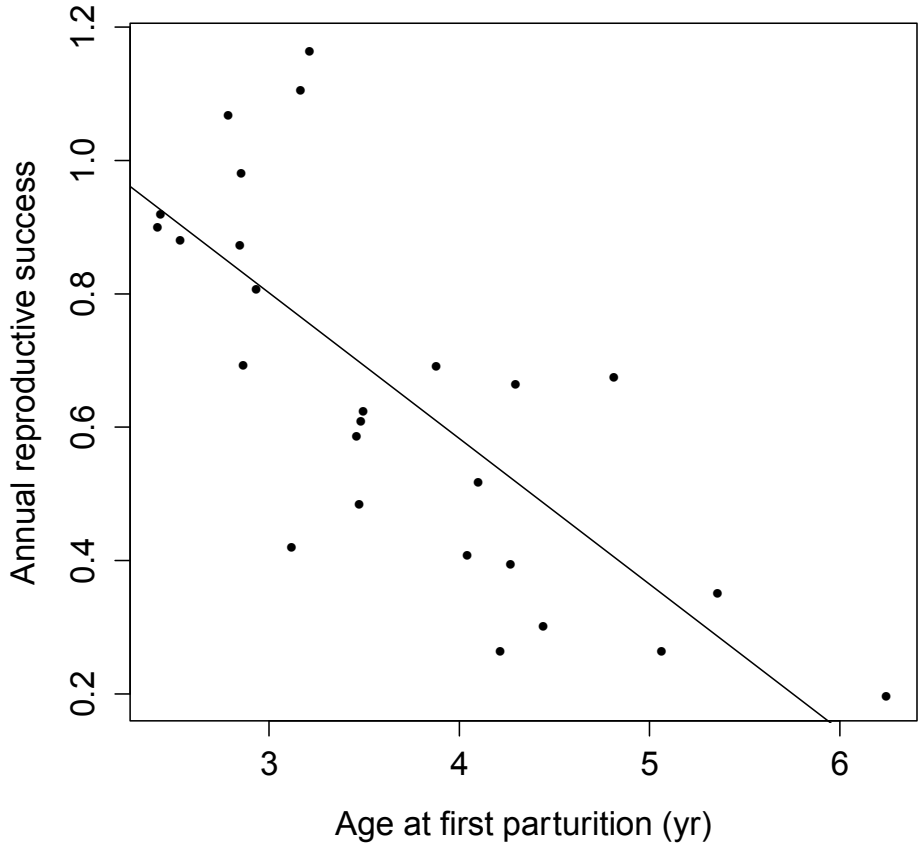

Figure B.3. Female spotted hyenas that were younger at first parturition experienced higher annual reproductive success than others (N = 26 females that survived past 3 years of age, successfully weaned at least one cub, and had known death dates; r^2 = 0.57; t_{24} = -5.61; P < 0.0001). A female's annual reproductive success was calculated as the number of her weaned cubs divided by her reproductive lifespan. Both female and male offspring are included.

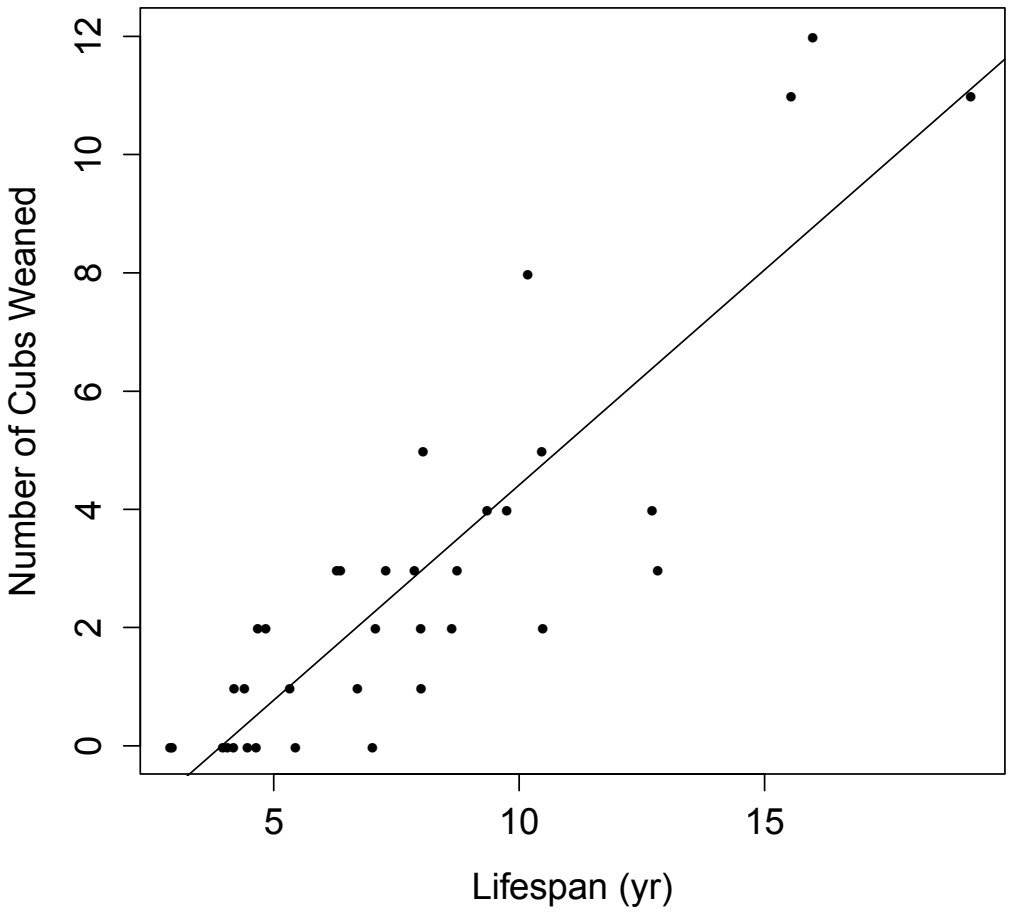

Figure B.4. Female spotted hyenas that lived longer weaned more cubs than those with relatively short lifespans (N = 35 females that survived past 3 years of age and with known death dates; r^2 = 0.76; t_{33} = 10.33, P <0.0001). Data points include five females that did not give birth to any offspring and four females that failed to wean any offspring. Both female and male offspring are included.

CHAPTER 4: INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) AS A POTENTIAL MEDIATOR OF FEMALE-BIASED SEXUAL SIZE DIMORPHISM IN WILD SPOTTED HYENAS

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Introduction

Sexual size dimorphism (SSD) occurs within species when males and females differ in their average adult body sizes (Fairbairn, Blanckenhorn & Szekely 2007). Among mammals, SSD is largely male-biased with sex-specific patterns of sexual selection explaining most of this variation (Darwin 1871; Ralls 1977; Lindenfors, Gittleman & Jones 2007). Male mammals often secure resources (e.g. mates, territories, etc.) by fighting, and larger males generally win these fights. Larger male body size is typically accompanied by sexually selected weapons or ornaments (Andersson 1994). Although less frequent than male-biased SSD, female-biased SSD does occur in mammals and, in contrast to this phenomenon in males, it rarely appears to be the consequence of sexual selection (Ralls 1976). The selective pressures favoring female-biased SSD are currently being explored (Schulte-Hostedde *et al.* 2002; Kilanowski & Koprowski 2016), but the mechanisms mediating its occurrence remain poorly understood.

Theoretically, the mechanisms that facilitate male-biased SSD should operate similarly in both male- and female-biased species due to high genetic overlap and the shared mechanisms that regulate growth (Badyaev 2002). Indeed, one of most remarkable aspects of SSD is that such sex-specific phenotypic diversity has evolved despite strong intersexual genetic correlations. Thus, understanding the proximate mechanisms mediating SSD has the potential, not only to reveal common targets of selection, but also to illuminate evolutionary solutions to problems posed by genetic constraints. Furthermore, mechanistic studies may also inform and stimulate hypotheses explaining the ultimate causes of both female- and male-biased SSD (Badyaev 2002; Bernstein 2010).

Although testosterone has been hypothesized to be the main driver of SSD, inconsistent results suggest that this relationship is neither direct nor universal across species (Jansson, Edén & Isaksson 1985; Sadighi *et al.* 2001; Taylor & Denardo 2005; John-Alder, Cox & Taylor 2007; Cox, Stenquist & Calsbeek 2009; Starostova *et al.* 2013). One major problem for this hypothesis is the failure of testosterone to explain female-biased SSD. For example, in the

spotted hyena (*Crocuta crocuta*), females are larger than males (Ralls 1976; Swanson *et al.* 2013), yet concentrations of testosterone are higher in developing males than in females (Glickman *et al.* 1987; Licht *et al.* 1992). A more plausible scenario might be that gonadal hormones act as sex-specific modifiers that orchestrate changes in shared anabolic pathways (Wade 1976; Holzenberger *et al.* 2001; Cox *et al.* 2017); however, it remains unknown whether the pathways associated with male-biased SSD are also associated with female-biased SSD.

The polypeptide hormone, insulin-like growth factor-1 (IGF-1) is a plausible mediator of SSD. Its positive direct effects on growth rates have been documented in both sexes among a variety of invertebrate and vertebrate taxa (Sonntag *et al.* 1999; Tatar *et al.* 2003; Kenyon 2010; Panici *et al.* 2010; Svensson *et al.* 2011; Yuan *et al.* 2012; Junnila *et al.* 2013; Bartke *et al.* 2013; Lewin *et al.* 2016). Although the liver is the primary source of circulating concentrations of IGF-1, most tissues of the body also produce IGF-1 (Cohick & Clemmons 1993). The dual endocrine and paracrine actions of IGF-1 allow it to direct coordinated and essential transformations during ontogenetic development (Cohick & Clemmons 1993; Holzenberger *et al.* 2003). Perhaps some of the strongest support for a role of IGF-1 in the mediation of SSD comes from studies of domesticated animals in which body size has been artificially selected (Gatford *et al.* 1998). For example, a single nucleotide polymorphism in the *IGF1* gene accounts for a substantial amount of variation in size between giant and small breeds of dogs (Sutter *et al.* 2007). Although work in free-living populations is limited, IGF-1 explained variation in size and life-history patterns among garter snakes (Sparkman *et al.* 2009, 2010), and a recent study of sexually dimorphic anole lizards revealed significant sex-biased expression in IGF-1 (Cox *et al.* 2017). These findings make IGF-1 a particularly intriguing candidate mechanism for mediating SSD in natural populations.

Here, we investigated IGF-1 as a potential mediator of female-biased SSD in the spotted hyena; our work had three specific goals. The first was to describe intra- and inter-sexual variation in circulating IGF-1 concentrations across the lifespan in this species. In spotted

hyenas the period of dimorphic growth is between 1 and 4 years of age, when females grow faster than males (Swanson *et al.* 2013); during the first year of postnatal life the sexes do not differ in size. If IGF-1 mediates the emergence of SSD in spotted hyenas, then we would expect to find sex differences in IGF1 concentrations during this dimorphic growth period. Profiles of IGF-1 concentrations across the lifespan are rare among free-ranging mammals, particularly for both sexes (but see & Kemnitz, 1992; Suzuki & Ishida, 2001), so our data should represent a substantial contribution to this literature.

Our second goal was to describe the sensitivity of postnatal IGF-1 concentrations to socio-ecological variables in both sexes. Postnatal development represents not only a time of significant size growth but also a potential sensitive window during which physiological processes may be capable of exerting long-lasting effects on later-life phenotypes and fitness (Bateson 1979; English *et al.* 2016). Theoretically, IGF-1 could mediate SSD through sex-specific sensitivities during postnatal development. For example, size disparities can result when a growth trait in one sex responds differently to an environmental stimulus than does the same trait in the opposite sex (Badyaev *et al.* 2001). Predicting which sex should be more or less sensitive is not straightforward (Stillwell *et al.* 2010), but the adaptive canalization hypothesis proposes one approach. Over time, the development of traits that are closely linked to fitness converges on an optimal “canal” leaving development more robust against fluctuating extrinsic conditions (Waddington 1942; Stearns & Kawecki 1994; Fairbairn 2005). If we were to extend this hypothesis to spotted hyenas, then we might expect juvenile IGF-1 concentrations in females to be more resistant to variation in socio-ecological variables than in males because female size is positively tied to fitness (Swanson *et al.* 2011; Lewin *et al.* 2016). Furthermore, our previous analysis of females revealed that juvenile IGF-1 concentrations did not vary with maternal rank, age, or prey density (Lewin *et al.* 2016), suggesting that IGF-1 might be canalized in females. However, whether and how IGF-1 concentrations might vary with environmental fluctuations among juvenile male hyenas remains unknown.

We selected socio-ecological variables likely to affect food intake because postnatal diet is known to influence IGF-1 concentrations (Isley, Underwood & Clemmons 1983; Breese, Ingram & Sonntag 1991; Clemmons & Underwood 1991; Kappeler *et al.* 2009). Juvenile hyenas, called cubs, are dependent on their mother's milk before weaning at around 1 year of age (Holekamp *et al.* 1996), so a mother's resources should be expected to affect her offspring's nutritional status (Hinde & Milligan 2011). Spotted hyenas live in groups structured by linear dominance hierarchies in which an individual's social rank determines its priority of access to food resources (Kruuk 1972; Tilson & Hamilton 1984; Frank 1986). As a result, maternal social rank may influence the IGF-1 concentrations in her developing offspring. Spotted hyenas typically bear litters of one or two cubs, and a cub's intra-litter dominance rank directly determines its access to milk (Frank, Glickman & Licht 1991; Smale *et al.* 1995; Wahaj & Holekamp 2006; Benhaiem *et al.* 2012). Effects of maternal and intra-litter dominance rank on juveniles are most evident during periods of low resource availability (Holekamp *et al.* 1996; Hofer & East 2003; Wahaj & Holekamp 2006) so we also considered local prey abundance as a variable that might affect IGF-1 concentrations. Finally, maternal parity can affect postnatal IGF-1 concentrations in some species (Hyatt et al. 2007; Bernstein et al. 2012) so we also took a mother's reproductive history into account.

Our final goal was to investigate the adaptive significance of sex differences in IGF-1 concentrations. Although male size in adulthood is not directly linked to reproductive fitness in hyenas (Höner *et al.* 2010), SSD might emerge through sexually dimorphic selection pressures operating during ontogeny (Lande & Arnold 1985; Badyaev *et al.* 2001; Karubian & Swaddle 2001; Schulte-Hostedde *et al.* 2002). Hyenas experience a period of intense selection (i.e. high mortality) after weaning but before reaching sexual maturity (Watts *et al.* 2009), during which SSD emerges in hyenas (Swanson *et al.* 2013). If patterns of IGF-1 underlie female-biased SSD in hyenas, we would expect size to have sexually dimorphic effects on survival to sexual maturity. Among juvenile females, circulating IGF-1 concentrations during the first year of

postnatal life correlate positively with relative juvenile mass, which predicts greater probability of survival to adulthood (Lewin *et al.* 2016). However, these relationships remain unstudied among juvenile males. If selection affects males and females differently early in life, then we expected to see a weaker relationship between juvenile size and survival among males than among females.

Materials and Methods

Field study site, subjects, and quantification of independent variables

Our subjects were part of a long-term field study of spotted hyenas within the Masai Mara National Reserve, Kenya. Behavioral, demographic and body size data were collected along with blood samples from members of three social group, or clans, called the Talek, Mara River, and Fig Tree clans. The Talek clan has been monitored continuously since 1988; data from the Mara River and Fig Tree clans have been collected since 2001 and 2007, respectively. Ages of hyenas in their natal clans were known to ± 7 days based on methods described earlier (Holekamp *et al.* 1996). We defined juvenile hyenas as those less than 1 year old. We estimated ages of immigrant males (± 6 months) using tooth wear data (Van Horn *et al.* 2003). Conception dates and reproductive states were calculated by subtracting the 110 day gestation period from parturition dates (Holekamp et al., 1996). We classified maternal parity as either 'primiparous' if the sampled cub was a member of the mother's first litter, or 'multiparous' if the cub was a member of a subsequent litter.

Within our study population, males typically disperse between 2 and 6 years of age (Smale *et al.* 1997; Boydston *et al.* 2005), but females are philopatric. Natal males were considered to have dispersed only if they had undertaken exploratory forays outside the natal territory, were in good health when last seen, and were last seen in the natal territory after 2 years of age (Van Horn *et al.* 2003). Otherwise males were considered to have died if they disappeared before they reached 2 years of age. We observed our study clans daily so we recorded a male's date last seen as its date of death.

As part of our long-term hyena field study, dominance rank matrices were constructed annually, based on outcomes of dyadic agonistic interactions (Smale *et al.* 1993). Here we calculated an adult hyena's within-sex relative rank as the proportion of adults of the same sex over which it was dominant during the year of sampling. We standardized social rank to account for variation in clan size at time of sampling; the individual with a relative rank of 1 was the highest-ranking animal in the clan at that time, and the individual with a relative rank of -1 was the lowest ranking. We assigned pre-reproductive females their mothers' standardized relative ranks at the time of their births. We determined intra-litter dominance rank based on outcomes of agonistic interactions between littermate siblings in twin litters (Smale *et al.* 1995). Cubs were then assigned as 'dominant twin', 'subordinate twin', or 'singleton' if they had no littermate.

The Masai Mara National Reserve is characterized by open, rolling grasslands, home to multiple resident ungulate species. For 3 to 4 months each year, large migratory herds of wildebeest (*Connochaetes taurinus*) and zebra (*Equus burchelli*) move into the Reserve creating a superabundance of available prey for spotted hyenas (Holekamp *et al.* 1997). Spotted hyenas appear to hunt whichever species is most abundant (Kruuk 1972; Cooper 1990; Hofer & East 1993b; Holekamp *et al.* 1997). We calculated average local prey density as the mean number of ungulates counted within 100 meters of multiple 4-km transect lines run through each clan's territory at biweekly intervals during the six months prior to collection of each blood sample.

We analyzed a total of 318 plasma samples collected between 1990 and 2014 from 271 unique individuals aged 0.5-15.0 years at the time of immobilization and blood sample collection. Hyenas younger than 0.5 years old could not be safely immobilized. As part of the longitudinal study, juvenile hyenas are routinely anaesthetized with Telazol (Zoetis, South Bend, IN; 6.5 mg/kg) administered in a plastic dart fired from a CO_2-powered rifle (Telinject Inc., Saugus, CA). All immobilizations were conducted early in the morning and all were carried out according to guidelines specified by the American Society of Mammalogists (Sikes 2016) and

approved by the Institutional Animal Care and Use Committee at Michigan State University (MSU; AUF #05/14-087-00). Blood samples were taken from the jugular vein in heparinized vacutainer tubes, centrifuged, and plasma was drawn off, aliquoted, then promptly placed in liquid nitrogen until it could be shipped on dry ice to Michigan State University, where samples were stored at -80˚C. Hyena body mass was recorded in kilograms (kg).

Quantification of plasma IGF-1 concentrations

We asssayed plasma IGF-1 concentrations using methods described previously (Lewin *et al.* 2016). Briefly, we quantified plasma IGF-1 levels using a commercially available enzyme immunoassay kit (EIA; ALPCO Diagnostics, Salem, NH). We validated this assay for spotted hyenas using the 'spike and recovery' method following kit protocol. All samples were run in duplicate and randomly assigned to plates with mean intra- and inter-assay coefficients of variation of 3.06% and 10.5%, respectively. Plasma samples were kept frozen at -80°C until analysis with the length of time frozen varying between 3 months and 24 years. We found no significant influence of freezer storage time ($r^2 < 0.001$, $t_{316} = -0.83$, $P = 0.41$).

Data analysis

Our dataset was composed of IGF-1 concentrations for 318 samples from 271 individuals varying in age and number of samples per individual, as some individuals were sampled multiple times. To compare differences in IGF-1 concentrations between the sexes, we used separate simple linear regressions among hyenas of five different age groups: 0-0.99 years, 1-1.99 years, 2-2.99 years, 3-3.99 years, and 4+ years. Where IGF-1 concentrations could not be normalized using traditional transformations we assessed sex differences using non-parametric Mann-Whitney-Wilcoxon tests. Residuals from all linear regression models satisfied assumptions of normality based on Shapiro-Wilk tests.

We used a two-tailed paired *t*-test on data from 9 females to compare within-individual IGF-1 concentrations between pregnancy and lactation. Where females had more than one sample per reproductive condition we took an average of their IGF-1 values in each condition.

Similarly, we used a paired *t*-test on 10 pairs of age-matched males to compare adult immigrant males with adult males still living in their natal territory; ages within these adult male pairs never differed by more than 12 months.

To determine the influence of juvenile IGF-1 concentrations on juvenile male mass, we built a multiple linear regression model using juvenile IGF-1, maternal rank, and prey density as predictor variables, similar to the model previously used in females (Lewin *et al.* 2016). Mass measurements were unavailable for 5 males; however, these individuals were included in all other analyses. To evaluate survival to reproductive maturity among males, we assessed male survival to 2 years of age with a Cox proportional hazards model identical to that previously used for analysis of survival among females in our study population (Lewin *et al.* 2016). All independent variables were mean centered with a standard deviation of 1.

To evaluate sensitivity of IGF-1 concentrations to socio-ecological variables during early postnatal life we built separate multiple linear regression models for females and males aged 0-0.99 years. Residuals from all linear regression models satisfied assumptions of normality based on Shapiro-Wilk tests. We used Tukey's post-hoc tests to identify significant differences among individuals based on intra-litter rank status. Non-significant interaction terms were removed from models to avoid overestimation (Bolker *et al.* 2009). To examine the effect of intra-litter dominance rank on IGF-1, we did a separate analysis with a two-tailed paired *t*-test looking at only twins sampled within two weeks of one another (15 litters: 3 male-male pairs, 4 female-female pairs, 8 female-male pairs; 8 litters where the subordinate twin was sampled first, 7 litters where the dominant twin was sampled first). All independent variables were mean centered with a standard deviation of 1.

Results

Variation in IGF-1 concentrations with age and sex

Both sexes showed a gradual decline in IGF-1 concentrations over the lifespan with the highest mean values seen in hyenas younger than 4 years old (Table C.1, Figure 4.1). Females

had significantly higher IGF-1 concentrations than did males in every age group (Table C.1) and across the growth period of 1-4 years of age (W = 4151, p = 0.0007). Among 9 females with IGF-1 concentrations measured during both pregnancy and lactation, IGF-1 concentrations were significantly higher during pregnancy than were those during lactation (t_8 = 4.52, P = 0.002; Figure C.1). A two-tailed paired t-test on 10 pairs of age-matched males revealed no significant difference in IGF-1 concentrations between immigrant and adult natal males (t_9 = -1.09, P = 0.30).

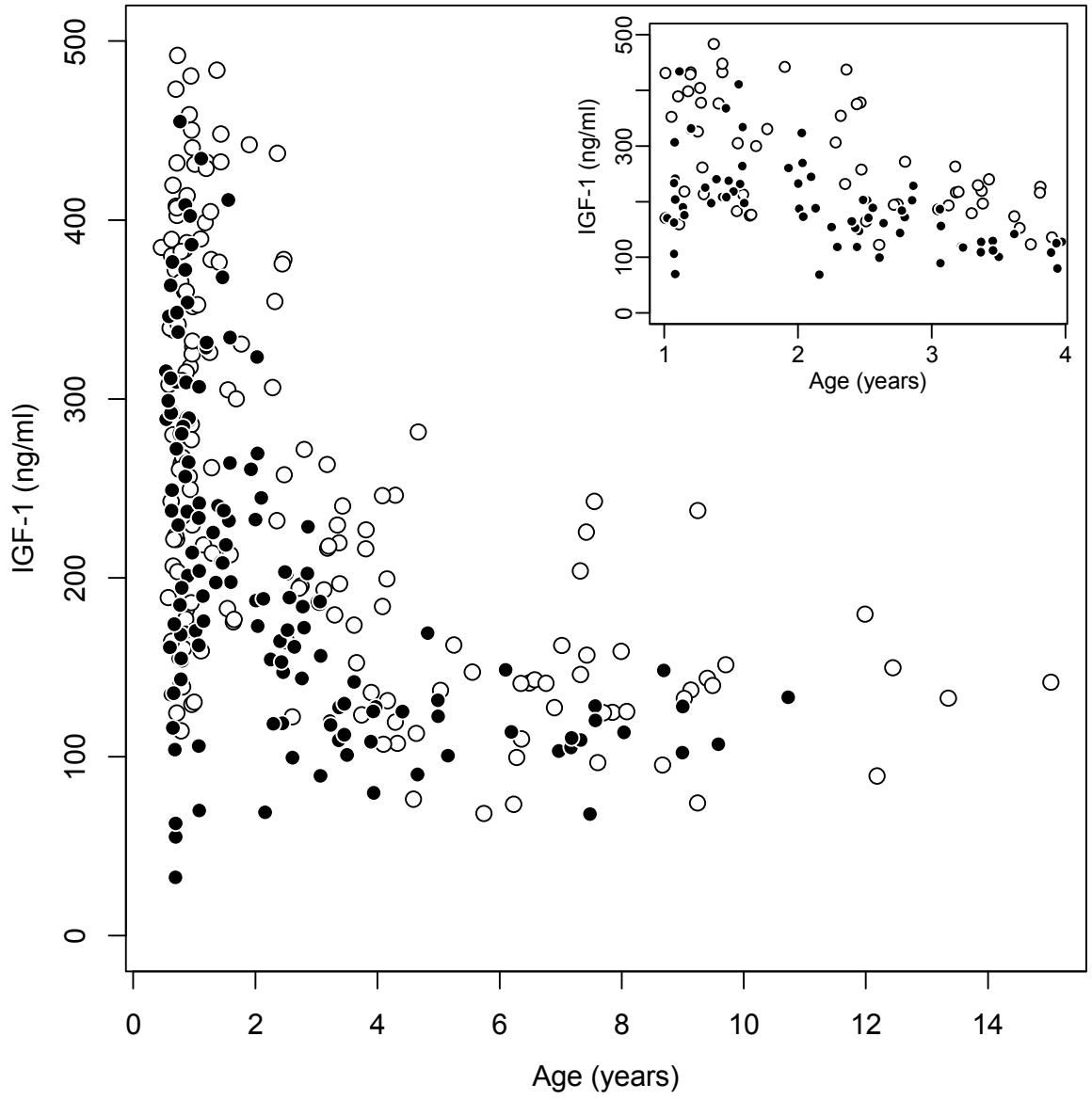


Figure 4.1. Patterns of circulating concentrations of IGF-1 across the lifespan in male (filled circles) and female (open circles) spotted hyenas. Inset is magnified between 1-4 years of age to better visualize the dimorphic growth period.

Sex-specific sensitivity of IGF-1 concentrations among males and females

Among juvenile males, maternal social rank, prey density, maternal parity, and intra-litter dominance rank significantly predicted IGF-1 concentrations (Table 4.2). Among 15 pairs of mixed-sex (n=8) and same-sex (n=7) twin littermates sampled within 2 weeks of one another, dominant twins had significantly higher concentrations of IGF-1 than did their subordinate littermates (two-tailed paired *t*-test: $t_{14} = 4.03$, $P = 0.001$). However, this relationship was not significant when we only looked at same-sex litters (two-tailed paired *t*-test: $t_6 = 1.76$, $P = 0.13$). Among juvenile females, we found no support for age, maternal social rank, prey density, maternal parity, or intra-litter dominance rank as significant predictors of IGF-1 concentrations (Table 4.3).

Table 4.1. Results from (a) multiple linear regression models to estimate influence of socio-ecological factors on IGF-1 concentrations among juvenile males, ages 0.5-1.0 years (n = 48). (b) Contrasts using least-squares means for intra-litter dominance rank. *P*-values indicating statistically significant differences at α = 0.05 are bolded.

(a) Model with singleton cub born to a multiparous mother set as the intercept.

Model fit: n = 48, adjusted R^2 = 0.22, p = 0.012

Fixed effect	**Parameter ± SE**	**95% CI**	***t***	**df**	***P***
Intercept	5.64 ± 0.10	5.44− 5.84	57.05	41	<0.0001
Age	0.95 ± 0.62	-0.30− 2.20	1.54	41	0.13
Maternal rank	**0.34 ± 0.13**	**-0.07 −0.61**	**2.54**	**41**	**0.01**
Intra-litter rank - dom	-0.10 ± 0.26	-0.62− 0.42	-0.40	41	0.69
Intra-litter rank - sub	**-0.41 ± 0.17**	**-0.74−-0.07**	**-2.45**	**41**	**0.02**
Maternal parity - primiparous	**-0.58 ± 0.18**	**-0.94−-0.22**	**-3.29**	**41**	**0.002**
Prey density	$1.1\times10^{-3} \pm 1.4\times10^{-3}$	-1.8×10^{-3}– 4.0×10^{-3}	0.77	41	0.45

(b) Same model as above with dominant twin set as intercept (all other estimates are identical).

Contrast	**Parameter ± SE**	**95% CI**	***t***	**df**	***P***
Intercept	5.56 ± 0.13	5.29− 5.83	41.37	41	<0.0001
Intra-litter rank - sub	-0.18± 0.17	-0.52− 0.16	-1.07	41	0.29
Singleton cub	0.11± 0.22	-0.33− 0.54	0.50	41	0.62

All continuous predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. Model residuals did not violate assumptions of normality (Shapiro-Wilk tests of normality: W ≥ 0.95, $P \geq 0.06$). Juvenile hyenas were assigned their mother's dominance rank (Maternal rank) at the time of immobilization; a relative rank of 1 was the highest-ranking animal in the clan, and the individual with a relative rank of -1 was the lowest ranking. Age was calculated in months using known birthdates. Maternal parity was coded as either primiparous or multiparous.

Table 4.2. Results from (a) multiple linear regression models to estimate influence of socio-ecological factors on IGF-1 concentrations among juvenile females, ages 0.5-1.0 years (n = 67). (b) Contrasts using least-squares means for intra-litter dominance rank. *P*-values indicating statistically significant differences at α = 0.05 are bolded.

(a) Model with singleton cub born to a multiparous mother set as the intercept.

Model fit: n = 67, adjusted R^2 = 0.002, p = 0.418

Fixed effect	**Parameter ± SE**	**95% CI**	***t***	**df**	***P***
Intercept	306.27 ± 25.93	254.39 − 358.15	11.81	60	<0.0001
Age	-33.04 ± 96.93	-226.93 − 160.85	-0.34	60	0.73
Maternal rank	-10.14 ± 23.52	-57.18 − 36.90	-0.43	60	0.67
Intra-litter-rank - dom	9.92 ± 30.83	- 51.76 − 71.59	0.32	60	0.75
Intra-litter-rank - sub	-60.46 ± 36.92	-134.30 − 13.38	-1.64	60	0.11
Maternal parity - primiparous	27.69 ± 36.39	-45.10 − 100.48	0.76	60	0.45
Prey density	0.17 ± 0.19	-0.21− 0.56	0.91	60	0.37

(b) Same model as above with dominant twin set as intercept (all other estimates are identical).

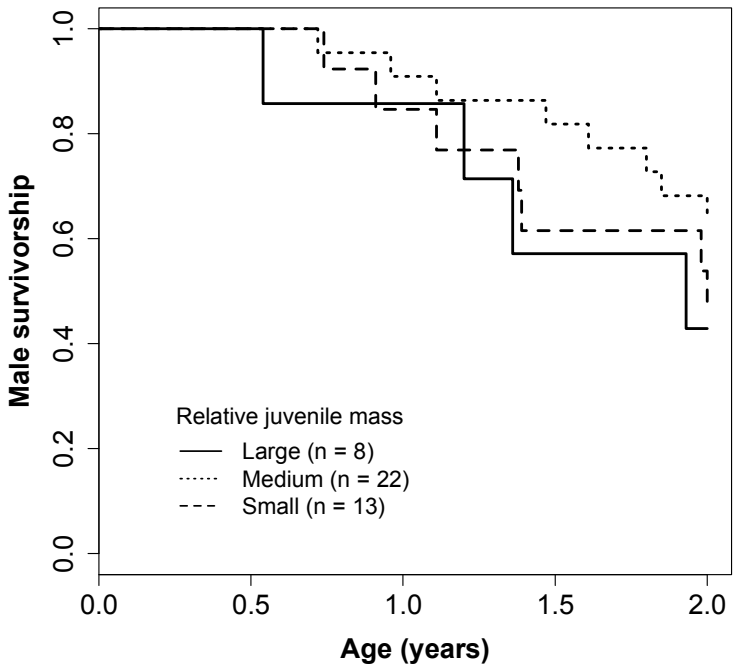
Contrast	**Parameter ± SE**	**95% CI**	***t***	**df**	***P***
Intercept	316.19 ± 16.98	282.23 − 350.15	18.65	60	<0.0001
Intra-litter rank - sub	-9.92 ± 30.83	-60.52 − 62.57	-0.32	60	0.75
Singleton cub	-70.38 ± 30.30	-127.16 − -4.43	-2.32	60	**0.02**

All continuous predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. Model residuals did not violate assumptions of normality (Shapiro-Wilk tests of normality: W ≥ 0.98, *P* ≥ 0.23). Juvenile hyenas were assigned their mother's dominance rank (Maternal rank) at the time of immobilization; a relative rank of 1 was the highest-ranking animal in the clan, and the individual with a relative rank of -1 was the lowest ranking. Age was calculated in months using known birthdates. Maternal parity was coded as either primiparous or multiparous.

Male survival to reproductive maturity

Juvenile IGF-1 concentrations significantly predicted relative juvenile mass among males (t_{39} = 5.15, P < 0.001; Table 4.4), but maternal rank and prey density did not (t_{39} = -1.76, P = 0.09; t_{39} = 1.44, P = 0.16, respectively; Table C.2). Neither relative juvenile mass (z = 0.05, P = 0.96) nor maternal rank (z = 0.83, P = 0.43) significantly predicted survival to reproductive maturity among males (Table C.3; Figure 4.2a), as it did for females (Figure 4.2b). Similarly, a model with juvenile IGF-1 as a covariate instead of juvenile mass also failed to predict male survival (z = -1.58, P = 0.12; Table C.3).

A



B

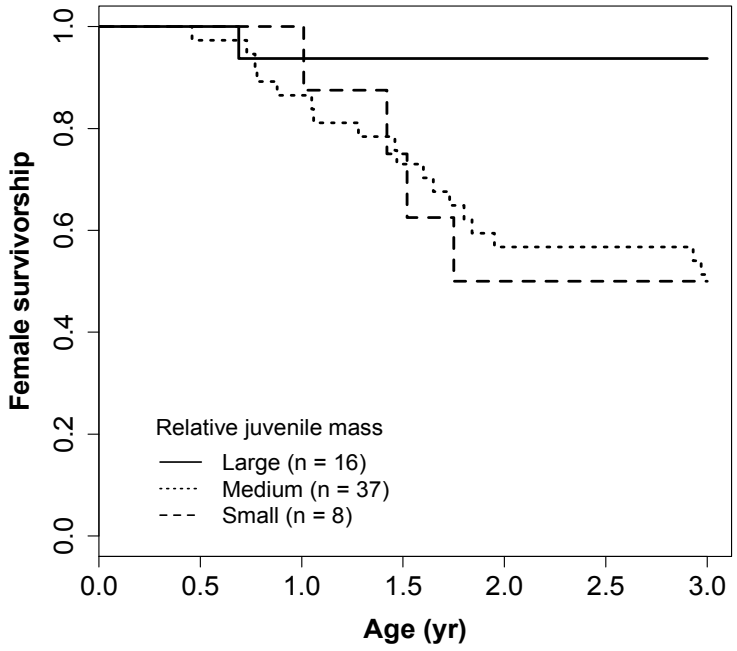


Figure 4.2. Juvenile mass did not predict survival to reproductive maturity in (A) males, but did so in (B) females. Relative mass was statistically analyzed as a continuous variable (Tables 4.4 & 4.5), but partitioned into tertiles for illustrative purposes only.

Discussion

In non-primate mammals, sexually dimorphic patterns of IGF-1 are typically male-biased where males also exhibit larger mean body mass than females (Gatford *et al.* 1998). In the present study, we found higher IGF-1 concentrations in the larger sex of a species displaying female-biased SSD (Table C.1, Figure 4.1), suggesting that similar physiological mechanisms may be responsible for dimorphic growth in species with either male- or female-biased SSD. Specifically, female spotted hyenas exhibited consistently higher IGF-1 concentrations than did males throughout the lifespan, starting as early as the first postnatal year of life (Table C.1; Figure 4.1). Interestingly, dimorphic growth does not occur until the second year of postnatal life (Swanson *et al.* 2013). Previous work in our study population confirmed that females grow faster than males between 1-4 years of age, rather than longer (Swanson *et al.* 2013), so our finding of higher IGF-1 concentrations in females just before and during the growth period implicates a role for IGF-1 in mediating sex-specific growth rates. Developing tissues may have sex-specific sensitivities to IGF-1 (Gatford *et al.* 1998; Holzenberger *et al.* 2001), but IGF-1 concentrations had similar slopes in the regression models predicting juvenile mass in males (Table C.2) and females (Table C.3). These results, along with the similar IGF-1 patterns observed across the lifespan in both sexes (Figure 4.1), suggest that sex differences in growth rate may be driven more by absolute IGF-1 concentrations than sex-specific sensitivities in target tissues.

Reproductive status appears to influence circulating IGF-1 concentrations among female, but not male, spotted hyenas. We observed significantly higher IGF-1 concentrations in pregnant female hyenas than in the same individuals when they were lactating (Figure C.2). The increase in IGF-1 levels before, and the subsequent decline after, parturition may be partially explained by the relationship between maternal IGF-1 and fetal growth. In humans, maternal IGF-1 levels positively correlate with placental size and surface area, thus potentially facilitating the exchange of nutrients between the mother and fetus (Liu *et al.* 1994; Chellakooty *et al.* 2004).

We found no significant difference in IGF-1 concentrations between age-matched natal and immigrant adult males suggesting that dispersal status alone does not appear to affect IGF-1 concentrations in male spotted hyenas. Among adult male spotted hyenas, post-immigration dispersal status is associated with higher concentrations of plasma testosterone than those found in adult males still living with their natal clans (Holekamp & Smale 1998; Holekamp & Sisk 2003; Dloniak *et al.* 2004). Experimental elevation of testosterone raises IGF-1 levels (Hobbs *et al.* 1993), so immigrant males would be expected to have higher IGF-1 concentrations than natal males if those concentrations were being mediated by T.

Maternal and familial variables played a significant role in predicting IGF-1 concentrations, but only among juvenile male hyenas. Specifically, juvenile males born to high-ranking, multiparous mothers exhibited the highest IGF-1 concentrations (Table 4.3). If food intake influences IGF-1 concentrations then the greater access to resources afforded to mothers of higher social rank should be expected to affect offspring concentrations. The differences in IGF-1 levels between primiparous and multiparous mothers suggests that experienced mothers may be better at acquiring resources and/or provisioning offspring. Interestingly, we did not find an effect of prey density on IGF-1 levels, which suggests that mothers may vary in how they mediate the effects of environmental conditions on their developing offspring. The distribution of maternal resources *via* litter size also influenced IGF-1 concentrations. Our within-sex analysis (Table 4.3) showed that subordinate male cubs displayed significantly lower IGF-1 concentrations than did singleton male cubs but not dominant male cubs. A failure to see significant differences between siblings of 7 same-sex litters also confirmed that litter size might be a more important factor in predicting IGF-1 concentrations than intra-litter rank.

Sex-biased sensitivity of growth traits to extrinsic factors has been reported in a range of wild vertebrate species exhibiting varying degrees of size differences between the sexes (Leberg & Smith 1993; Sheldon *et al.* 1998; Post *et al.* 1999; Toïgo *et al.* 1999; Badyaev *et al.* 2001; Kalmbach, Furness & Griffiths 2005; Råberg, Stjernman & Nilsson 2005; Verhulst,

Holveck & Riebel 2006; Wilkin & Sheldon 2009; Hinde 2009; Bernstein *et al.* 2012). In many of these studies, the precise divergence and duration of male and female growth patterns during postnatal development are believed to contribute largely to the realized difference, or degree, of SSD observed in adulthood. For example, in red deer (*Cervus elaphus*), female and male growth rates responded differently to shared environmental conditions leading to greater degrees of SSD during periods of warmer climate and smaller degrees in periods of varying food abundance (Post *et al.* 1999). The adaptive canalization hypothesis seeks to explain sex-biased trait sensitivity further by predicting greater trait robustness in the sex for which the studied trait is positively linked to fitness (Stearns & Kawecki 1994; Fairbairn 2005). Because juvenile IGF-1 concentrations correlated positively with larger juvenile mass and higher juvenile survival among female hyenas, we predicted that females would be the more robust sex. Consistent with this prediction, plasma IGF-1 concentrations of juvenile females did not vary with environmental variables (Table C.3), whereas those of juvenile males did so (Table C.2).

Sex-biased sensitivity and canalization may be one possible outcome when the selective optimum for a trait differs between the sexes (Ralls 1977; Lande 1980; Price 1984), such that any explanation of male- or female-biased SSD should accommodate not only why one sex is larger but also why the other sex is smaller (Hedrick & Temeles 1989). Here, we found sex-specific relationships between juvenile mass and survival to reproductive maturity. Among males, we saw a significant relationship between IGF-1 and relative mass (Table C.2), but neither juvenile IGF-1 nor juvenile mass predicted survival to reproductive maturity (Table C.4; Figure 4.2A). In contrast, among female spotted hyenas, maternal rank and higher concentrations of IGF-1 predicted larger relative juvenile mass (Table C.3), which in turn predicted greater probability of survival to reproductive maturity (Table C.5; Figure 4.3B).

Previous research on our study population found evidence of positive selection on size traits in male hyenas as well as in females, although it was considerably stronger on females than males (Swanson 2013). The author used annual reproductive success as a measure of

fitness whereas we focused on survival to reproductive maturity so our results are not in conflict. Rather, taken together our results present a more complex picture and suggest that size may confer different advantages before and after reproductive maturity. Currently, we can only speculate why mass is more important to survival among juvenile females than juvenile males, but sex-biased survivorship is common in the wild (Schulte-Hostedde *et al.* 2002; Kalmbach *et al.* 2005; Råberg *et al.* 2005; Bonduriansky *et al.* 2008; Maklakov *et al.* 2008). Low probability of survival among small males might be due to small males losing to larger siblings in food contests, whereas low probability of survival among large males might be due to the costs of maintaining a large body size, such as higher nutritional demands (Clutton-Brock, Albon & Guinness 1985; Arendt 1997; Blanckenhorn 2000; Dmitriew 2011).

Due the opportunistic and sensitive nature of immobilizing hyenas in our study population, we cannot definitively rule out the contributions of IGF-1 outside the ages of our analyses. Specifically, the period during which development is more sensitive to environmental conditions may be sexually dimorphic (Clutton-Brock *et al.* 1985; Gatford *et al.* 1998; Lindstrom 1999; Badyaev 2005) so it is possible that the IGF-1 system in female hyenas indeed varies with environmental conditions, but outside our analysis period, which focuses on the first postnatal year of life. Similarly, IGF-1 levels and mass among juvenile male hyenas may be sensitive to environmental variables throughout ontogeny and not limited to the age range studied here. For example, in contrast to previous findings in a Tanzanian population of spotted hyenas (Höner *et al.* 2010), maternal rank did not significantly predict relative male mass during the first postnatal year of life in our study area (Table C.2). However, the Tanzanian study measured mass until the age of 6 months whereas the youngest male cub in our study was 7 months old, so the contribution of maternal rank to body mass in earlier periods of development is certainly possible. In either case, our results provide support for postnatal concentrations of IGF-1 as physiological mediators of SSD in spotted hyenas, and suggest that neutral selection

for male juvenile mass and positive selection for female juvenile mass might be one potential route by which SSD is maintained in this species.

APPENDIX

Supplementary Material for Chapter 4

Table C.1. Distribution of samples, excluding 12 samples from 9 pregnant females (mean IGF-1 ± s.e. (ng/ml) = 176.89 ± 15.98). With the sexes pooled over the growth period of 1-4 years of age, females displayed significantly higher IGF-1 concentrations than males (W = 4151, p = 0.0007).

Age range	Male mean IGF-1 ± s.e. (ng/ml)	No. samples, individuals	Female mean IGF-1 ± s.e. (ng/ml)	No. samples, individuals	Result of model IGF-1 ~ Sex
0-1	247.63 ± 14.58	48, 48	302.29 ± 11.97	68, 68	W = 2092, p = 0.01*
1-2	242.94 ± 16.28	27, 27	324.31 ± 20.70	26, 26	t_{51} = -3.10, p = 0.003
2-3	182.10 ± 10.61	26, 26	273.99 ± 26.06	12, 12	t_{36} = -3.75, p < 0.001
3-4	122.24 ± 6.78	15, 14	198.21 ± 9.57	16, 16	t_{29} = -6.40, p < 0.001
4+	118.09 ± 4.80	21, 21	157.47 ± 10.45	47, 40	W = 683, p = 0.01*

*IGF-1 concentrations could not be normalized using traditional transformations so we assessed sex differences using a non-parametric Mann-Whitney-Wilcoxon Test.

Table C.2. Results from a multiple linear regression model estimating influence of socio-ecological factors on relative juvenile mass among 43 juvenile males (ages 0.5-1.0 years). Mean mass ± SE = 18.90 ± 0.77 kg. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Model fit: n = 43, adjusted R^2 = 0.39, p < 0.0001

Fixed effect	**Parameter ± SE**	**95% CI**	***t***	**df**	***P***
Intercept	-1.85×10^{-16} ± 0.4	-0.88 − 0.88	0.00	39	1.00
Juvenile IGF-1	**2.25 ± 0.4**	**1.36 − 3.17**	**5.15**	**39**	**<0.0001**
Maternal rank	-0.78 ± 0.4	-1.70 − 0.12	-1.76	39	0.09
Prey density	0.62 ± 0.4	-0.30 − 1.48	1.44	39	0.16

All predictor variables were z-transformed to a mean of 0 and standard deviation of 1. Model residuals did not violate assumptions of normality (Shapiro-Wilk test of normality: W = 0.99, P = 0.86).

Table C.3. Results from a multiple linear regression model estimating influence of socio-ecological factors on relative juvenile mass among 69 juvenile females (ages 0.5-1.0 years). Mean mass ± SE = 22.88 ± 0.74 kg. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Fixed effect	Parameter ± SE	95% CI	*t*	df	*P*
Intercept	-7.54 ± 1.90	-11.3 − 3.7	-3.96	65	**<0.001**
Juvenile IGF-1	**0.02 ± 0.00**	**0.01 − 0.03**	**3.80**	**65**	**<0.001**
Mat. dom. rank	**2.67 ± 0.93**	**0.82 − 4.53**	**2.89**	**65**	**0.005**
Prey density	0.01 ± 0.01	-0.01 − 0.02	1.23	65	0.224

All predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. Model residuals did not violate assumptions of normality (Shapiro-Wilk test of normality: W = 0.99, *P* = 0.75).

Table C.4. Results from a Cox proportional hazards model predicting male survival to reproductive maturity. Males of larger relative juvenile mass between 0.5 and 1.0 years did not survive proportionally better or worse than smaller males. Model 2 was the best-supported model based on log likelihood with a higher value indicating a better fitting model.

Model 1: n= 43; χ^2 =0.63; df = 2, *P* = 0.73, model log likelihood = -65.85

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile mass	0.01	1.01	0.05	0.96
Maternal rank	-0.19	0.83	-0.79	0.43

Model 2: n= 43; χ^2 = 3.05; df = 2, *P* = 0.22, model log likelihood = -64.60

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile IGF-1	-0.38	0.68	-1.58	0.12
Maternal rank	-0.15	0.86	-0.57	0.57

All predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. A negative hazard rate coefficient (β) indicates a decreased probability of survival with increasing value of the predictor variable. A positive hazard rate coefficient (β) indicates an increased probability of survival with increasing value of the predictor variable. A hazard ratio (calculated as *exp*(β)) of 2, for example, indicates that individuals are twice as likely to survive with increasing values of the predictor variable.

Table C.5. Results from two separate Cox proportional hazards models predicting female survival to reproductive maturity. Heavier juvenile female hyenas experienced significantly greater survival to reproductive maturity than did smaller females. Model 2 was the best-supported model based on likelihood ratio tests. One individual had no mass recorded so was not included in Model 2, in which mass was a predictor variable; however, this individual did have an IGF-1 value so was included in Model 1. Significant effects are highlighted in boldface at a significance level of $\alpha = 0.05$.

Model 1: n= 62; $\chi^2 = 3.29$; df = 2, $P = 0.19$, model log likelihood = -88.59

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile IGF-1	-0.32	0.72	-1.46	0.145
Mat. dom. rank	-0.40	0.67	-1.06	0.288

Model 2: n= 61; $\chi^2 = 6.20$; df = 2, $P = 0.45$, model log likelihood = -86.67

Predictor variable	**β**	**Hazard ratio**	***z***	***P***
Juvenile mass	**-0.11**	**0.90**	**-2.27**	**0.023**
Mat. dom. rank	-0.07	0.93	-0.17	0.868

All predictor variables were *z*-transformed to a mean of 0 and standard deviation of 1. A negative hazard rate coefficient (β) indicates a decreased probability of survival with increasing value of the predictor variable. A hazard ratio (calculated as *exp*(β)) of 2, for example, indicates that individuals are twice as likely to survive with increasing values of the predictor variable.

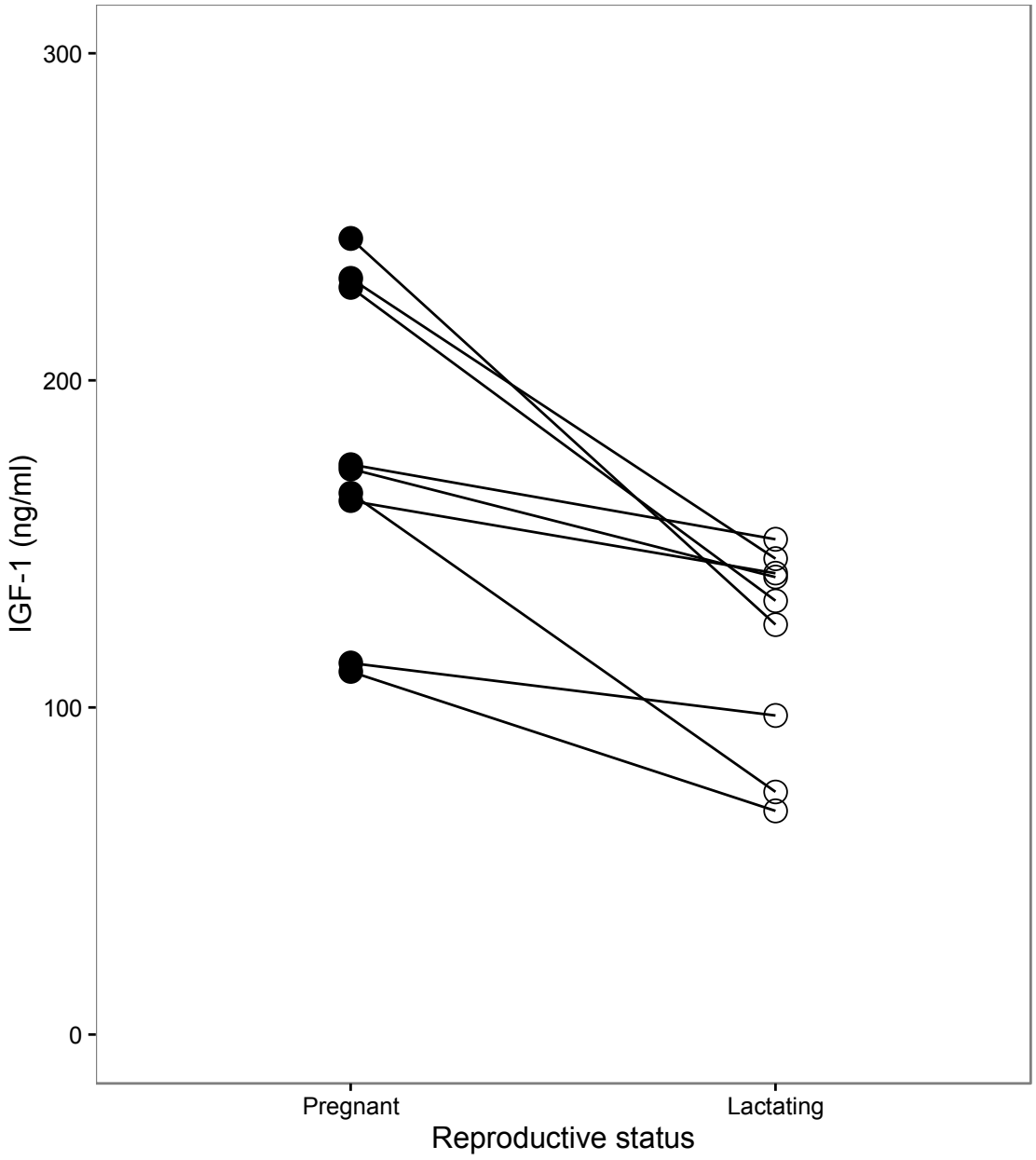


Figure C.1. Plasma IGF-1 concentrations were significantly higher in females while pregnant (solid circles) than while lactating (open circles). Two-tailed paired *t*-test: $t_8 = 4.53$, $P = 0.002$. Lines represent individual female subjects (n = 9 females).

CHAPTER 5: AGE-RELATED REPRODUCTIVE PERFORMANCE VARIES WITH DOMINANCE RANK AMONG FEMALE SPOTTED HYENAS

Introduction

As detailed data accumulate from long-term, individual-based field studies on iteroparous species, increasingly more studies are documenting, not only how reproductive performance improves over the lifespan, but also how it declines among the oldest animals in natural populations (Bronikowski & Promislow 2005; Robbins *et al.* 2006; McCleery *et al.* 2008; Nussey *et al.* 2008a, 2013; Reed *et al.* 2008; Dugdale *et al.* 2011; Roach & Carey 2014; Hammers *et al.* 2015; Lee *et al.* 2016). Evolutionary theories of aging were originally based upon data from experimental laboratory studies (Stearns 1992; Bronikowski & Promislow 2005), so the influx of empirical data offers the exciting opportunity to complement this groundbreaking work with a deeper understanding of how age-related reproductive performance has evolved in response to, or in spite of, environmental variation (Williams *et al.* 2006).

Currently, two non-mutually exclusive hypotheses have been offered to explain age-related improvement and decline in reproductive performance. Early in life, age-related improvement in reproductive performance may best be explained by the constraint hypothesis (Forslund & Pärt 1995). Under this hypothesis, reproductive fitness should increase as developmental constraints (e.g. physiological immaturity, lack of experience rearing young) are removed, allowing for improvement in traits important for offspring survival. For example, many female mammals begin reproducing before reaching full adult body size, which creates an energetic conflict between the needs for maternal growth and offspring survival (Trivers 1972; Stearns 1989). However, as a female matures and the energy required for her growth decreases, she is free to allocate more resources toward the survival of her offspring, thus increasing her realized reproductive success. The constraint hypothesis may also apply to pre- and postnatal experience rearing young. Inexperienced mothers may not know how to properly provision or attend to offspring, particularly in species lacking communal rearing of offspring (Wang & Novak 1994; Cameron *et al.* 2000).

Late-life declines in reproductive performance are often explained by the senescence hypothesis, which predicts age-related declines in reproductive performance (Medawar 1952; Hamilton 1966; Charlesworth 1993). Under the senescence hypothesis, declines in reproductive performance are the realized physiological consequences of investing energy resources into reproduction rather than into self-maintenance earlier in life. To date, two non-mutually exclusive hypotheses have been proposed to explain declines in reproductive performance. The disposable soma theory explains the declines in reproductive performance as the erosion of essential physiological functions by the lifelong accumulation of cellular damage (Kirkwood & Holliday 1979), whereas the theory of antagonistic pleiotropy stipulates that the same genes associated with fitness benefits early in life are linked to negative consequences (i.e. senescence) later in life (Williams 1957). Regardless of which mechanism is operating, both generate similar predictions for the optimal allocation of limited energy, with trade-offs being a central feature (Kirkwood & Rose 1991).

Competition among conspecifics may influence the reproductive patterns exhibited within a population by restricting individual access to food resources and mating opportunities (Clutton-Brock 1988; Holekamp *et al.* 1996; Altmann & Alberts 2003). In many animal societies structured by rigid linear hierarchies, individuals occupying the top dominance ranks enjoy priority of access to both, and, as a consequence, also enjoy superior reproductive performance (Clutton-Brock, Albon & Guinness 1986; Holekamp *et al.* 1996; Creel *et al.* 1997; Pusey, Williams & Goodall 1997; Hofer & East 2003; Sharp & Clutton-Brock 2011). Despite the ubiquity of this relationship, relatively few studies have explored the degree to which rank-related performance is attributable to earlier improvements and/or delayed senescence (Holekamp *et al.* 1996; Wasser *et al.* 1998; Côté & Festa-Bianchet 2001; Von Holst *et al.* 2002; Watts *et al.* 2009; Sharp & Clutton-Brock 2010; Hoffman *et al.* 2010).

Here, we use 27 years of individual-based, longitudinal data from a population of spotted hyenas (*Crocuta crocuta*) to test for constraint and senescence in several measures of

reproductive performance. Unlike other social mammals in which dominant individuals monopolize breeding (Clutton-Brock & Huchard 2013), all female hyenas are free to breed year-round with equal access to mates (Kruuk 1972), making them a unique system in which to study age- and social rank-related variation in reproduction. Previous research in this population shows that high-ranking females start reproducing at younger ages (Holekamp *et al.* 1996) and so should be expected to show earlier improvements in reproductive performance than would low-ranking females (Watts *et al.* 2009). However, it remains unknown whether earlier reproductive improvements are accompanied by earlier reproductive declines, and, if so, how this might vary with social rank across the lifespan.

Materials and Methods

Our subjects were drawn from a long-term field study of a single large social group of spotted hyenas, the Talek clan, which has been monitored continuously since May 1988 within the Masai Mara National Reserve, Kenya. Clan size has varied over time (Holekamp *et al.* 2012) ranging from around 42 to 126 individuals (Green 2015). The home territory occupied by this clan is primarily open, rolling grassland with permanent water and year-round prey availability. All hyenas in the study clan were identified by their unique spot patterns, and sex was identified based on the morphology of the glans of the erect phallus (Frank et al. 1990). Demographic and behavioral data were collected as part of year-round, routine observations of the Michigan State University (MSU) Hyena Project started in 1988 by Dr. Kay E. Holekamp and Dr. Laura Smale.

The gestation period in the spotted hyena is 110 days (Kruuk 1972). Females typically give birth to small litters containing only one or two cubs, which they nurse for up to 24 months (Holekamp *et al.* 1996). A female typically bears her litter in a solitary natal den before transferring the cubs to a communal den used by other clan members when her cubs are 2-5 weeks old. Birthdates of cubs were estimated to ± 7 days based on their pelage, size, and other aspects of their behavior and appearance when first seen above ground (Holekamp *et al.* 1996).

We determined age at first parturition based on the initial tearing of the female's pseudopenis, indicated by the presentation of pink scar tissue on its posterior surface (Frank & Glickman 1994). This tearing reliably indicates that first parturition has occurred regardless of whether or not the first litter born is ever seen above ground by observers.

Dominance rank matrices were constructed based on outcomes of dyadic agonistic interactions (Tilson & Hamilton 1984; Frank 1986). We standardized rank to account for variation in clan size; an individual with a relative rank of 1 was the highest-ranking animal in the clan, and the individual with a relative rank of -1 was the lowest ranking. High-ranking females were considered those with relative ranks of 1 to 0.33, mid-ranking females were those with relative ranks of 0.33 - 0.33, and low-ranking females were those with relative ranks of -0.33 to -1. Each female was assigned a rank in the adult female hierarchy upon the birth of her first litter or her third birthday, whichever came first.

Female age at last parturition and lifespan

A female's age at last parturition was calculated as her age when she gave birth to her most recent litter if she was still alive, or last recorded litter if she was deceased. Because female spotted hyenas are philopatric (Smale et al 1997; Boydston et al 2005), and because we were observing our study clans daily, we recorded each female's date last seen as her date of death.

Lifetime reproductive success (LRS)

We calculated lifetime reproductive success as the total number of cubs a female successfully weaned during her lifespan. We only calculated LRS of females that gave birth to at least one litter and had full reproductive histories with known death dates.

Age of cub at weaning

We estimated the age at weaning to within 10 days using observations of weaning conflicts between a mother and her cubs (Holekamp *et al.* 1996). A singleton litter was defined

as a litter from which only one cub successfully weaned, regardless of whether neonatal litter size was one or two; both cubs were successfully weaned in twin litters.

Inter-birth interval (IBI)

We calculated the inter-birth interval as the number of months between consecutive parturitions when at least one cub survived to weaning (singleton) or both cubs survived to weaning (twin litter). If one member of a twin litter died before 6 months of age, but the remaining cub survived to weaning, then we counted the litter as a singleton litter.

Cub survival

In our study population, males typically disperse between 2 and 6 years of age (Smale *et al.* 1997; Boydston *et al.* 2005), but females are philopatric. Natal males were considered to have dispersed only if they had undertaken exploratory forays outside the natal territory, were in good health when last seen, and were last seen in the natal territory after 2 years of age (Van Horn *et al.* 2003). Males were considered to have died rather than dispersed if they disappeared before they reached 2 years of age. We observed our study clans daily so we recorded a male's date last seen as its date of death unless it was known to have dispersed.

Statistical analyses

We performed all analyses with R statistical software (Version 3.3.2; R Core Team 2016). For linear mixed effects models (LMM), we used the 'lme4' (Bates *et al.* 2014) package with *p*-values estimated by Satterthwaite's approximation for denominator degrees of freedom using the 'lmerTest' package (Kuznetsova, Brockhoff & Christensen 2015). For survival analyses, we used the mixed effects Cox model in the 'coxme' package (Therneau 2015).

LMMs offer a powerful statistical framework to separate between- and within-individual variation in traits (van de Pol & Verhulst 2006; Nussey *et al.* 2008a, 2013). Here, we included female identity (ID) and litter birth year as random effects to control for individual variation in reproductive performance and year-to-year environmental variation, respectively (Nussey *et al.* 2008a). All models (LMM, multiple linear regression, and mixed effects Cox) were initially fitted

with a full model of four fixed effects: female rank, female age, an interaction term (female rank × age) to accommodate potential age variation in rank-related reproduction, and (female age)^2 to accommodate a potential nonlinear relationship between reproduction and age (i.e. both improvement and decline in performance). When the interaction term or quadratic term was insignificant, we removed it as a fixed effect in a reverse stepwise model simplification method leaving only the linear terms of female rank and age. We then re-ran the model using restricted maximum-likelihood methods (Bolker *et al.* 2009). Residuals for all LMM and multiple linear regressions were normally distributed based on the Shapiro-Wilk test of normality ($W \geq 0.99$, $p \geq 0.35$; Burnham & Anderson 2002).

To analyze LRS, we performed a multiple linear regression. To analyze age at weaning and inter-birth interval, we performed LMMs with Gaussian distributions.

Results

Generally, female hyenas of all ranks reproduced close to the end of their lifespans and stopped producing young at around 17 years of age (Figure 5.1). At least eight of 93 deceased female hyenas in our study population have lived past 16 years of age with one individual living until 23 years of age (Figure 5.1), but most females live much shorter lifespans in the wild. Mean ± s.e. lifespan among deceased females was 8.25 ± 0.72 years for high-ranking females; 8.72 ± 0.78 years for mid-ranking females; and 8.81 ± 0.89 years for low-ranking females.

During our 27 years of data collection, high-ranking females produced the greatest number of litters with at least one cub surviving to weaning (Figure 5.2, Table 5.1). High-ranking females appeared to benefit from high early performance, with over 50% of 113 weaned litters born to mothers aged 4 years or younger, and peak performance at 3 years of age (Figure 5.2, Table 5.1). Mid-and low-ranking females also experienced peaks of performance, but at 4 and 5 years of age, respectively, likely owing to older ages at first parturition (Holekamp *et al.* 1996).

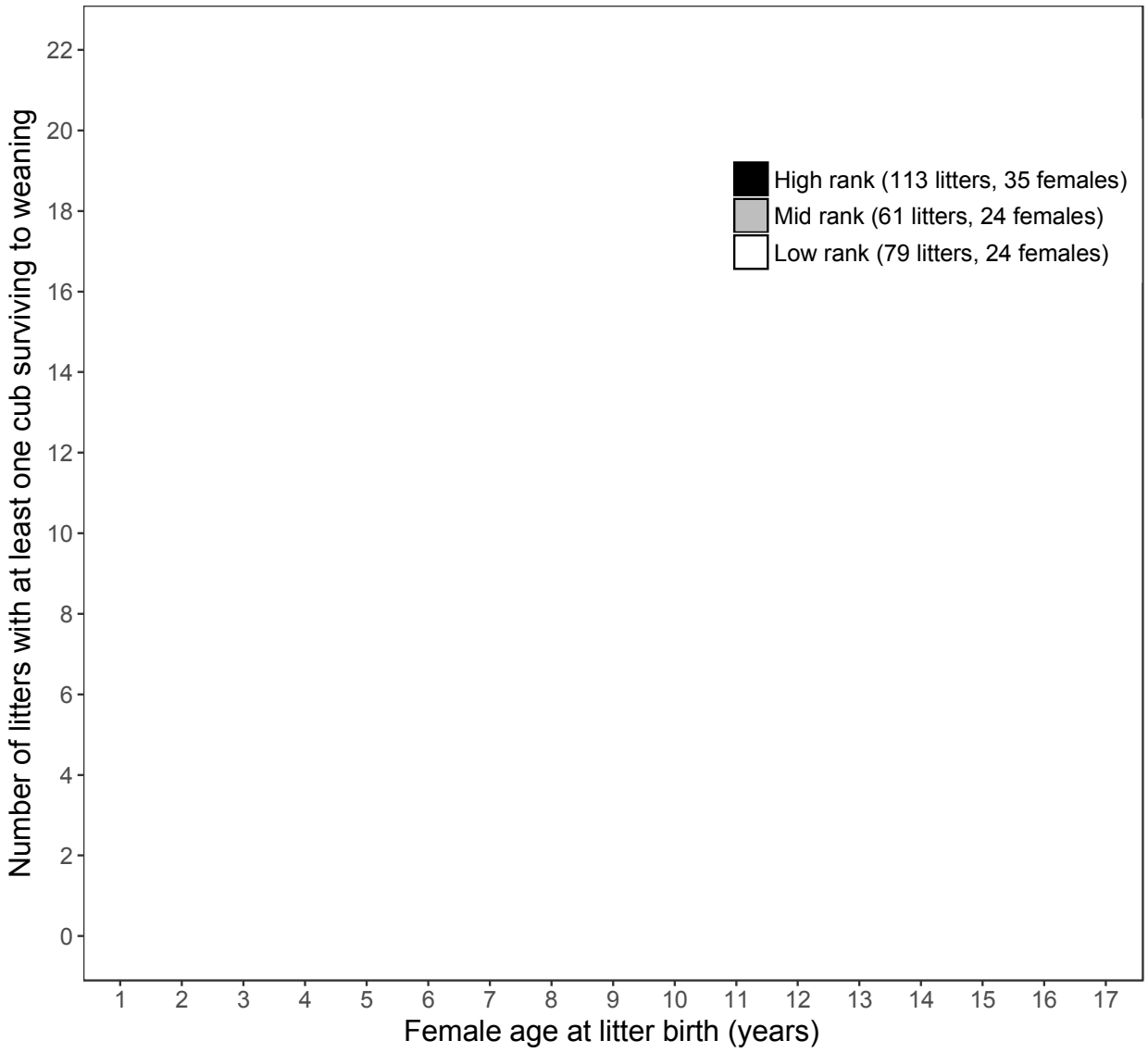


Figure 5.1. The number of litters with at least one cub surviving to weaning varied with binned maternal age (rounded down to the nearest integer value) and maternal rank (n =253 litters born to 83 unique female hyenas across 27 years). The total number of cubs that survived born to high-ranking females was 136, with 74 born to mid-ranking females, and 71 born to low-ranking females. Female rank was analyzed as a continuous variable, but is shown here as a categorical variable for illustrative purposes only. For example, the sole female in the 1-year category gave birth when she was 1.88 years.

Lifetime reproductive success (LRS)

We analyzed LRS for 48 deceased females, all of which were adults but 12 of which never successfully weaned any offspring (1 high-ranking, 5 mid-ranking, and 6 low-ranking females). Mean ± s.e. LRS among all females was 3.24 ± 0.52 cubs. Mean ± s.e. LRS was 4.00 ± 0.85 cubs for high-ranking females, 2.53 ± 0.85 cubs for mid-ranking females, and 2.93 ± 1.12 cubs for low-ranking females. Higher female rank and longer lifespans significantly predicted greater female LRS (rank: $t_{46} = 3.38$, $P \leq 0.001$; lifespan: $t_{46} = -10.04$, $P \leq 0.001$; Figure 5.3, Table 5.3).

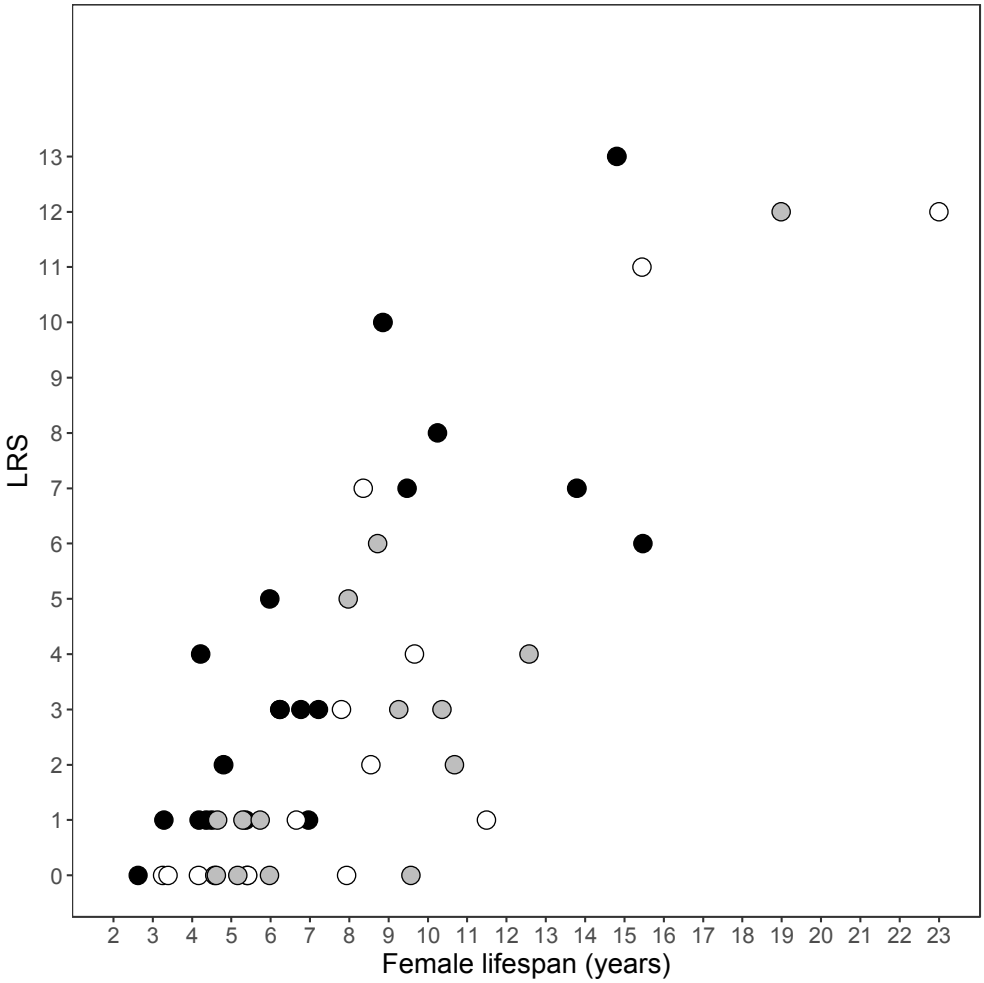


Figure 5.2. Lifetime reproductive success (LRS) of female spotted hyenas that had given birth to at least one litter and that had full reproductive histories with known death dates (high-rank: n = 17, mid-rank: n = 15, low-rank: n = 14). LRS was measured as the total number of cubs weaned; both male and female offspring are included. Shading denotes binned female rank (black = high, gray = mid, white = low), which was statistically analyzed as a continuous variable but shown here as a categorical variable for illustrative purposes only.

Age of cub at weaning - singleton

We analyzed cub age at weaning for 125 singleton cubs born to 60 females across 27 years. Mean ± s.e. cub age at weaning among high-ranking females was 10.51 ± 0.43 months, 12.82 ± 0.52 months for mid-ranking females, and 11.86 ± 0.47 months for low-ranking females. Female ID, but not cub birth year, explained a significant portion of the residual variation (X^2 = 13.05, P <0.001; Table 5.2), suggesting individual differences in mothering styles among breeding females. Of the fixed effects, neither (female rank × age) nor (female age)^2 was significant so both variables were eliminated from the final model. Neither female rank ($t_{54.0}$ = -1.47 P = 0.15) nor female age ($t_{120.6}$ = 1.63 P = 0.11) significantly predicted cub age at weaning (Table 5.3).

Age of cub at weaning - twin litter

We analyzed cub age at weaning for 76 twin litters cubs born to 40 females across 24 years. Mean ± s.e. cub age at weaning was 11.45 ± 0.38 months for high-ranking females, 11.99 ± 0.74 months for mid-ranking females, and 13.46 ± 0.73 months for low-ranking females. As was true for weaning age among singletons, female ID, but not cub birth year, explained a significant portion of the residual variation (X^2 = 7.59, P = 0.006; Table 5.2). (Female age)^2 was not significant so was eliminated from the final model. Younger ages at weaning were significantly predicted by higher female rank ($t_{73.6}$ = -2.30, P = 0.02) and (female rank **×** age) ($t_{73.6}$ = 2.16, P = 0.03; Table 5.3), but not by age *per se*.

Inter-birth Interval (IBI) after singleton litter

We analyzed the IBI following the successful weaning of 73 singleton litters born to 40 females across 25 years. Mean ± s.e. IBI was 14.98 ± 0.69 months for high-ranking females, 18.19 ± 0.62 months for mid-ranking females, and 17.79 ± 0.84 months for low-ranking females. Neither random effect explained a significant portion of the residual variation ($X^2 \leq 0.64$, $P \geq 0.11$; Table 5.2). Of the fixed effects, neither (female rank × age) nor (female age)^2 was significant, so both variables were eliminated from the final model. IBI was significantly

predicted by female rank ($t_{35.2}$ = -3.44, P = 0.002), but not by female age ($t_{69.3}$ = 0.32, P = 0.75; Figure 5.4A, Table 5.3) with high-ranking females exhibiting the shortest IBIs.

Inter-birth Interval (IBI) after twin litter

We analyzed the IBI following the successful weaning of 53 twin litters born to 28 females across 22 years. Mean ± s.e. inter-birth interval was 13.87 ± 0.72 months for high-ranking females, 18.64 ± 0.90 months for mid-ranking females; and 17.26 ± 1.31 months for low-ranking females. Neither random effect explained a significant portion of the residual variation ($X^2 \leq 0.70$, $P \geq 0.40$; Table 5.2). Of the fixed effects, neither (female rank × age) nor $(\text{female age})^2$ was significant, so both variables were eliminated from the final model. Older females showed significantly longer IBIs ($t_{46.6}$ = 3.59, P = 0.0008; Figure 5.4B, Table 5.3), but we saw no significant effect of female rank ($t_{28.7}$= -0.35, P = 0.73).

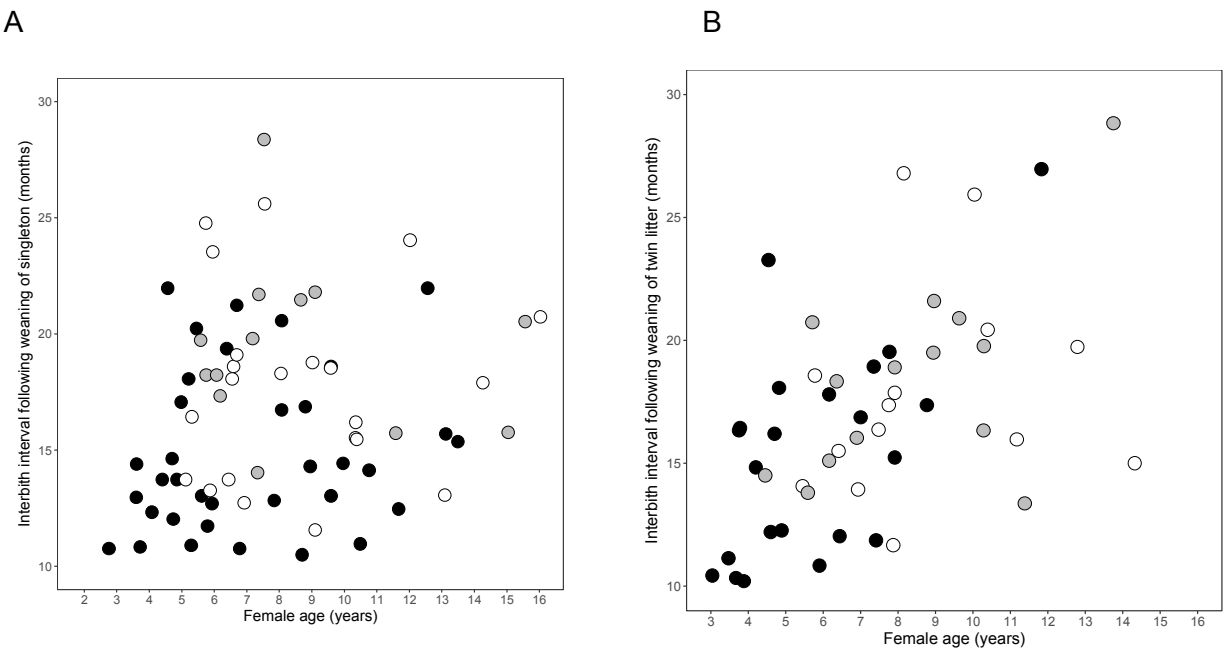


Figure 5.3. (A) Inter-birth intervals following successful weaning of singleton litters (n=72 litters born to 39 females; mean inter-birth interval ± s.e. for high-ranking females: 14.88 ± 0.69 months, for mid-ranking females: 18.59 ± 0.62 months, and for low-ranking females: 17.79 ± 0.84 months). (B) Inter-birth intervals following successful weaning of twin litters (n=53 litters born to 28 females). Shading denotes female rank (black = high, gray = mid, white = low). Female rank was statistically analyzed as a continuous variable but shown here as a categorical variable for illustrative purposes only.

Cub survival

We analyzed cub survival to 2 years of age among 670 cubs born to 112 females across 27 years. Mean ± s.e. number of cubs that survived was 3.32 ± 0.45 cubs born to high-ranking females, 2.39 ± 0.34 cubs born to mid-ranking females, and 2.73 ± 0.30 cubs born to low-ranking females. Total number of cubs that survived born to high-ranking females: 136 cubs, to mid-ranking females: 74 cubs, and to low-ranking females: 71 cubs. The random effect of cub birth year explained a significant portion of the residual variation ($X^2 = 26.76$, $P < 0.001$; Table 5.2) whereas female ID did not ($X^2 = 2.88$, $P = 0.09$; Table 2). Greater cub survival was significantly predicted by higher female rank ($z = -3.14$, $P = 0.002$) and older female age ($z = -2.07$, $P = 0.04$), and by (female rank × age) ($z = 2.19$, $P = 0.03$), and $(\text{female age})^2$ ($z = 2.25$, $P = 0.03$; Table 5.4), suggesting both age-related improvement and decline in performance.

Discussion

In rank-structured societies, an individual's rank may have an enormous effect on its ability to acquire resources and, consequently, on its fitness. Superior priority of access to food and mating opportunities generally affords high-ranking individuals high reproductive performance, but whether this competitive edge persists over the entire lifespan is less well understood. Here, we report both significant rank- and age-related improvement and decline in reproductive performance among female spotted hyenas occupying all ranks positions within the social hierarchy, and ranging in age from 3 to 23 years. Our findings suggest that reproductive senescence does occur in this species and that, in general, high-ranking females senesce earlier than do lower-ranking females. Interestingly, however, this does not prevent high-ranking females from out-performing lower-ranking females, as their LRS was nevertheless 25% greater than that of mid- or low-ranked females.

The constraint hypothesis (Forslund & Pärt 1995) predicts age-related improvement in reproductive performance at the beginning of an individual's reproductive lifespan. Across the study period, we saw that females of all dominance ranks experienced age-related increases in

the frequency and weaning success rate of litters containing at least one cub that survived to weaning (Figure 5.2, Table 5.1). Higher-ranking females start breeding at earlier ages (Holekamp *et al.* 1996), and, accordingly, we saw that they enjoyed high weaning success earlier in life with the largest number of weaned litters born to 3-year old females (Figure 5.2; Table 5.1). Mid- and lower-ranking females weaned far fewer litters early in life with the largest numbers of weaned litters born to 4- and 5-year old females, respectively (Figure 5.2; Table 5.1). Low-ranking females showed the greatest improvement with age; success rates doubled from 30% at age 3 to 60% at age 4 and peaked around 79% at age 7 (Table 5.1). However, the selective disappearance of low-performing individuals may give the false impression of improvement (Forslund & Pärt 1995; van de Pol & Verhulst 2006), and the reduction in the number of reproducing low-ranking females from ages 3 to 4 and from ages 6 to 7 (Table 5.1) certainly makes this plausible. Nevertheless, our results show that at least some low-ranking females are capable of high reproductive success. Additionally, the reproductive peaks for each rank group suggest different time points at which females may break free from developmental constraints, such as negative energy balance (Zehr, Van Meter & Wallen 2005) or immature levels of ovarian hormones (Van Meter *et al.* 2008). Future research coupling energetic and endocrine measures with behavioral data could illuminate not only the mechanisms of developmental constraint but also potentially those of reproductive senescence (i.e. menopause).

We found evidence of both reproductive improvement and senescence with advancing female age in only one measure: cub survival (Table 5.4). Our results mirror patterns found in some other mammals and birds (Sharp & Clutton-Brock 2010; Stahler *et al.* 2013; Hammers *et al.* 2015), suggesting that female hyenas follow similar age-related trends in offspring survival to those found in other taxa. Although it remains unclear how this is directly mediated, our finding of significantly longer IBI with advancing female age following successful weaning of twin litters may offer some indirect clues. Specifically, the linear trend of increasing IBI with female age

(Figure 5.4B; Table 5.3) coupled with the quadratic relationship of cub survival with female age (Table 5.4) suggests that cub survival may benefit from prolonged IBI, but only to a certain point. At younger ages, longer IBIs may indicate greater maternal investment (Paul, Kuester & Podzuweit 1993; Hoffman *et al.* 2010), but, at older ages, a longer IBI may indicate senescence in reproductive resilience, energetic condition, or fertility (Altmann *et al.* 2010; Nenko & Jasienska 2013). We only calculated IBI for females whose previous litters were successfully weaned so our results could be biased towards older, successful females that were able to both wean twin litters and live longer by virtue of elongating their IBI. However, female ID did not explain a significant portion of the residual variation here (Table 5.2), indicating that all females experienced this age-related increase in IBI following successful weaning of twin litters.

Although we saw a significant effect of maternal age following the successful weaning of twin litters, we did not see the same relationship when we looked at weaning of singletons. Here, instead of maternal age, maternal rank significantly predicted IBI following the successful weaning of singletons (Figure 5.4A; Table 5.3), which is consistent with earlier findings in this population (Holekamp *et al.* 1996). Our observation of age-related increases in IBI, but only after weaning twin litters, suggests that there may be larger trade-off between self-maintenance and reproduction as females move closer to senescence. Spotted hyenas are estimated to have the highest energy output during lactation among terrestrial carnivores (Oftedal & Gittleman 1989) so older females may be undergoing a more severe trade-off between self-maintenance and reproduction where the latter is prioritized. Given the enormous energetic toll of lactation, it may be no surprise that we observed longer recovery times in older females following successful weaning of twin litters but not singletons.

Cubs born to high-ranking females were weaned at earlier ages than were cubs born to low-ranking females (Table 5.3), but the significant interaction of (female rank × age) reported in this study suggests that this relationship varies with maternal age (Table 5.3). Specifically, the age at weaning for cubs of high-ranking females increased with maternal age thereby reducing

the disparity among ranks. Despite having an under-developed feeding apparatus, cubs of high-ranking females are able to wean at younger ages largely due to their mothers' aggressive displacement of conspecifics from food (Engh *et al.* 2000; Watts *et al.* 2009). Therefore, a prolonged lactation period among cubs of older high-ranking females may indicate senescence in the mother's ability to competitively secure resources for her cub. Because maternal support exerts an enormous effect on cub survival (Watts *et al.* 2009) it may be no coincidence that we also saw a significant interaction effect of between female rank and age on cub survival (Table 5.4).

Although the onset of reproductive senescence varied with dominance rank, all females ceased reproduction after 17 years (Figure 5.1, Table 5.1), suggesting a terminal point of reproduction in this species. However, we did observe a small percentage of female hyenas outliving their ability to reproduce by up to 7 years (Figure 5.1). These females varied in dominance status and age at last parturition, suggesting that the mechanisms contributing to post-reproductive lifespans may not be dictated by dominance rank. The occurrence of females with post-reproductive lifespans is rare among other mammalian species (Cohen 2004) and may be a random add-on at the end of the life history (Reznick, Bryant & Holmes 2006). Nevertheless, in killer whales (Foster *et al.* 2012) and elephants (Lee *et al.* 2016), the presence of post-reproductive females in kin groups directly contributes to offspring survival, potentially through leadership, caring for descendant kin, and the dissemination of knowledge regarding food resources (Brent *et al.* 2015) and threats from predators (McComb *et al.* 2011), respectively. It remains to be explored in spotted hyenas whether these post-reproductive females also contribute to their descendants' survival, or whether their long lives merely allow them to persist in the population without making contributions to the welfare of their descendants.

Although some high-ranking females live long lives, most need not have long lifespans to achieve LRS superior to that of subordinate females. For example, in order to achieve the

same LRS as that of high-ranking females, it may take some mid- and low-ranking females twice as many years (Figure 5.3). Despite the low weaning rate among mid- and low-ranking females, some females do achieve LRS that is similar to or greater than that of high-ranking females by virtue of living longer (Figure 5.3), thus highlighting the relative importance of longer lifespans to the fitness of lower-ranking females. Long lifespans have the potential to drive population dynamics in wild bats (Schorcht, Bontadina & Schaub 2009) and Soay sheep (Coulson *et al.* 2006), so exactly how they may operate in rank-structured societies with multiple matrilines is worthy of future research.

Our mixed effects modeling approach revealed significant individual variation among females with respect to the age at which their cubs weaned, but not for any other reproductive traits (Table 5.2). Although weaning is typically around 13 months of age in our study population of spotted hyenas (Holekamp et al 1996), it can range from 7.5 to 24 months and appears to be largely determined by maternal behavior and food availability (Holekamp *et al.* 1996) rather than achievement of a fully mature feeding apparatus (Watts *et al.* 2009). The degree of individual variation may depend on the life history of the study species (van de Pol & Verhulst 2006). Here, we attribute the lack of significant inter-individual variation among the other traits to the large amount of variation explained by female rank and age in this species. In other social species where dominance rank is more subject to change, we might expect more individual heterogeneity in reproductive performance particularly in traits that may facilitate both reproductive success and dominance acquisition (e.g. aggressive behavior, individual physical quality).

Life-history theory predicts that individuals that start reproducing early and exhibit high rates of reproduction should also experience early senescence (Williams 1957; Kirkwood & Rose 1991). Like alpha individuals in other hierarchical societies (Clutton-Brock *et al.* 1986; Creel *et al.* 1997; Pusey *et al.* 1997; Young, Carlson & Monfort 2006), alpha female hyenas show uniquely high reproductive performance within a clan (Holekamp *et al.* 1996). However,

contrary to life-history theory, alpha females appear to resemble “Darwinian demons” (Law 1979), which manage to escape fitness trade-offs; that is, alpha female hyenas are able to enjoy both early, high reproductive success and long lifespans. The average female hyena weans 3 cubs over an 8-year lifespan, but we recorded one alpha female weaning 13 cubs over a 15-year (Figure 5.3) and another weaning at least 13 cubs over a 16-year lifespans (data not shown; female was born before 1988 so we only have a partial reproductive history). It is likely that the high resource availability (Kruuk 1972; Tilson & Hamilton 1984), low markers of physiological stress (Lewin *et al.* 2015), and high immunity (Flies *et al.* 2016) afforded to alpha females allow them to escape the early senescence predicted by theory. Alternatively, there may be costs incurred by high-ranking females that we have yet to identify.

APPENDIX

Supplementary Material for Chapter 5

Table D.1. Summary table of 426 litters born to 113 unique female hyenas using over 27 years of longitudinal data. The % of total litters born with at least one cub surviving to weaning is rounded to the nearest integer value. This % was only calculated for age bins where ≥ 5 litters were recorded. Maternal rank was statistically analyzed as a continuous variable but shown here as a categorical variable for illustrative purposes only.

	Female social rank								
	High			Mid			Low		
Female age at litter birth (years)	# of litters born	# of litters with ≥1 cub surviving to weaning (% of total)	# of repro-ducing females	# of litters born	# of litters with ≥1 cub surviving to weaning (% of total)	# of repro-ducing females	# of litters born	# of litters with ≥1 cub surviving to weaning (% of total)	# of repro-ducing females
1	1	1	1	0	0	0	0	0	0
2	25	18 (72%)	25	6	4 (67%)	6	3	1	3
3	31	22 (71%)	26	20	5 (25%)	18	23	7 (30%)	22
4	26	17 (64%)	23	19	11 (58%)	18	15	9 (60%)	15
5	22	12 (55%)	20	10	7 (70%)	10	18	13 (72%)	17
6	18	12 (67%)	17	10	6 (60%)	9	17	10 (59%)	16
7	11	5 (45%)	10	12	8 (67%)	12	14	11 (79%)	13
8	12	9 (75%)	11	6	4 (67%)	6	8	5 (63%)	8
9	10	5 (56%)	9	7	4 (57%)	6	10	7 (70%)	10
10	5	3 (60%)	5	5	2 (40%)	5	8	6 (75%)	8
11	4	2	4	6	4 (67%)	6	5	1 (20%)	5
12	4	3	4	2	1	2	5	3 (60%)	5
13	3	2	3	5	2	4	2	1	2
14	4	1	1	1	0	1	4	3	4
15	1	1	1	3	3	3	1	1	1
16	1	0	1	0	0	0	2	1	2
17	0	0	0	1	0	1	0	0	0
Total:	178	113 (63%)		113	61 (54%)		135	79 (59%)	
Mean:	10.47	6.64	9.47	6.65	4.12	6.29	7.94	4.64	7.71

Table D.2. Effects of the random effects of female identity (ID) and offspring birth year in five separate linear mixed-effects models measuring reproductive performance. Random effects that explained a significant portion of the residual variance in each model are highlighted in boldface at a significance level of $\alpha = 0.05$.

Trait	Random effect	Variance	X^2	*P*
Singleton cub age at weaning	**Female ID**	**3.70**	**13.05**	**<0.001**
125 cubs born to 60 females across 27 years	Cub birth year	0.87	3.55	0.06
Twin cub age at weaning	**Female ID**	**2.65**	**7.59**	**0.006**
76 cubs born to 40 females across 24 years	Cub birth year	1.55	2.15	0.14
Inter-birth interval after singleton	Female ID	2.60	1.27	0.26
70 litters born to 38 females across 25 years	Cub birth year	1.00	0.32	0.57
Inter-birth interval after twin	Female ID	1.05	0.17	0.68
53 litters born to 28 females across 22 years	Cub birth year	1.77	0.70	0.40
Cub survival to 2 years	Female ID	0.06	2.88	0.09
690 cubs born to 126 females across 33 years	**Cub birth year**	**0.21**	**26.76**	**<0.001**

Table D.3. Results from models that best predicted reproductive performance in 5 traits. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Trait	Variable	Estimate	SE	df	*t*	*P*
Lifetime reproductive success	**Intercept**	**-2.39**	**0.62**	**46**	**-3.85**	**<0.001**
	Female rank	**1.70**	**0.50**	**46**	**3.38**	**<0.001**
	Female lifespan	**0.69**	**0.70**	**46**	**10.04**	**<0.001**
Age of cub at weaning - singleton	**Intercept**	**10.87**	**0.66**	**116.88**	**16.50**	**<0.0001**
	Female rank	-0.80	0.54	54.04	-1.47	0.15
	Female age	0.14	0.08	120.61	1.63	0.11
Age of cub at weaning - twin litter	Intercept	13.41	1.12	69.71	11.96	**<0.0001**
	Female rank	**-3.25**	**1.41**	**73.63**	**-2.30**	**0.02**
	Female age	-0.09	0.13	74.41	-0.66	0.51
	Female age × rank	**0.36**	**0.17**	**71.26**	**2.16**	**0.03**
Inter-birth interval after weaning singleton	**Intercept**	**16.75**	**1.30**	**67.66**	**12.86**	**<0.0001**
	Female rank	**-2.81**	**0.82**	**35.21**	**-3.44**	**0.002**
	Female age	0.05	0.15	69.25	0.32	0.746
Inter-birth interval after weaning twin litter	**Intercept**	**10.38**	**1.91**	**41.98**	**5.45**	**<0.0001**
	Female rank	-0.36	1.03	28.70	-0.35	0.73
	Female age	**0.85**	**0.24**	**46.61**	**3.59**	**0.0008**

Table D.4. Results from the best model predicting cub survival to 2 years. Significant effects are highlighted in boldface at a significance level of α = 0.05.

Trait	Variable	β	Hazard ratio	z	*P*
Cub survival to 2 years	**Female rank**	**-0.66**	**0.51**	**-3.14**	**0.002**
	Female age	**-0.17**	**0.85**	**-2.07**	**0.04**
	Female age × rank	**0.06**	**1.06**	**2.19**	**0.03**
	Female age^2	**0.01**	**1.01**	**2.25**	**0.03**

A negative hazard rate coefficient (β) indicates a decreased probability of survival with increasing value of the predictor variable. A hazard ratio (calculated as *exp*(β)) of 2, for example, indicates that individuals are twice as likely to survive with increasing values of the predictor variable.

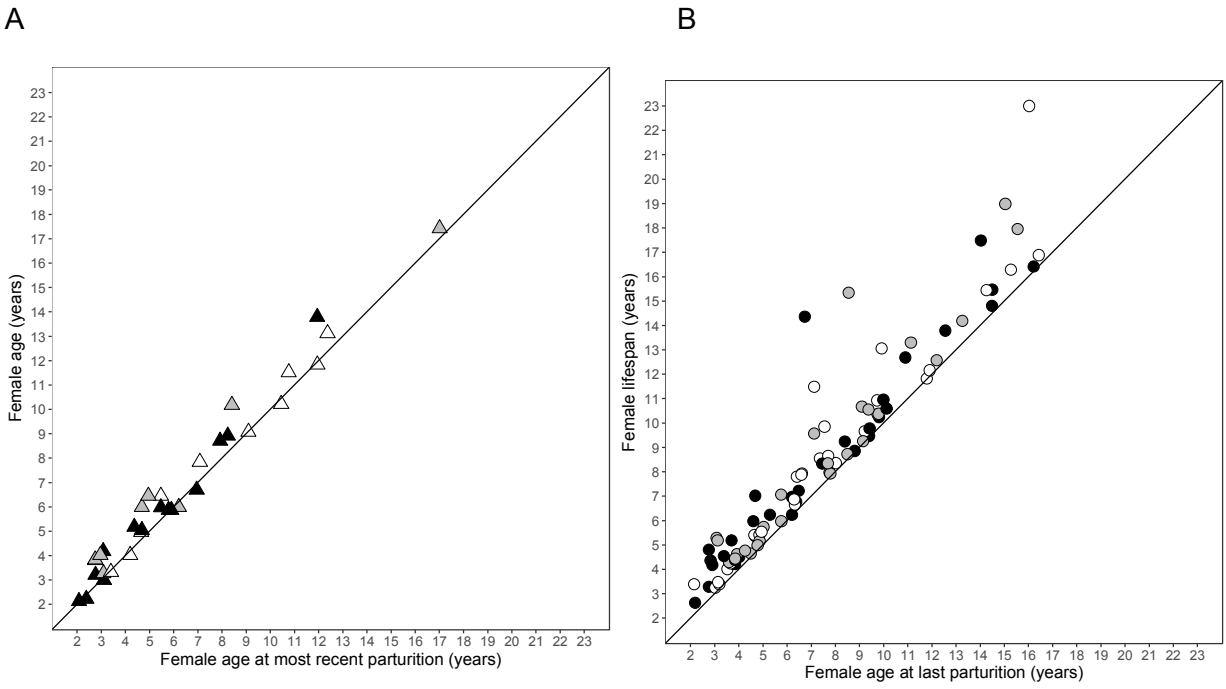


Figure D.1. Female age at the birth date of her last known litter regardless of whether or not it was successfully weaned. (A) Females still alive by the end of the study period (high-rank: n = 14, mid-rank: n = 8, low-rank: n = 10), with age at the end of the study period on the y-axis and age at most recent parturition on the x-axis. (B) Deceased females with known death dates that had given birth to at least one litter (high-rank: n = 34, mid-rank: n = 29, low-rank: n = 30), with lifespan on the y-axis and age at last recorded parturition on the x-axis. Shading denotes binned female rank (black = high, gray = mid, white = low). A point lying on the solid reference line (intercept = 0, slope = 1) indicates that the female died the same year as she last gave birth.

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