SIBLING RIVALRY IN THE SPOTTED HYENA (*Crocuta crocuta*): BEHAVIOURAL MECHANISMS AND ENDOCRINE RESPONSES

Inaugural-Dissertation to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy
of the Freie Universität Berlin

by

SARAH LEA KATRIN BENHAIEM
from Hamburg

Berlin, 2012

Diese Dissertation wurde am Leibniz-Institut für Zoo- und Wildtierforschung in Berlin (Direktor: Prof. Dr. Heribert Hofer) im Zeitraum September 2007 bis März 2012 unter der Leitung von Dr. Marion L. East und Prof. Dr. Heribert Hofer angefertigt und am Institut für Biologie der Freien Universität Berlin eingereicht.

1. Gutachter: Prof. Dr. Heribert Hofer
2. Gutachter: Dr. Christian Voigt

Disputation am: 05.06.2012

This dissertation is based on the following manuscripts:

1. Benhaiem S[1], Hofer H[1], Kramer-Schadt S[1], Brunner E[2] and East ML[1] (in revision) Sibling rivalry: training effects, emergence of dominance and incomplete control.

2. Benhaiem S[1], Dehnhard M[1], Bonnani R[1,3], Hofer H[1], Goymann W[4], Eulenberger K[5] and East ML[1] (minor revisions) Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in spotted hyenas (*Crocuta crocuta*).

3. Benhaiem S[1], Dehnhard M[1], Hofer H[1], Helms J[1] and East ML[1] (manuscript) Sibling rivalry in spotted hyenas (*Crocuta crocuta*) is stressful.

[1] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

[2] Department of Medical statistics, Humboldtallee 32, D-37073 Goettingen, Germany

[3] Dipartimento di Biologia Evolutiva e Funzionale, Università degli Studi di Parma, via G.P. Usberti 11/A, 43100 Parma, Italy

[4] Max-Planck-Institut für Ornithologie, D-82319 Seewiesen, Germany

[5] Zoo Leipzig GmbH, Pfaffendorfer Str. 29, D-04105 Leipzig, Germany

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

Berlin, den 30.03.2012

Sarah Benhaiem

CONTENT

CHAPTER 2 – MANUSCRIPT 1

CHAPTER 3 – MANUSCRIPT 2

CHAPTER 4 – MANUSCRIPT 3

ACKNOWLEDGEMENTS

I would like to thank those who made this thesis possible:

Above all, I am deeply thankful to my supervisors Dr. Marion L. East and Prof. Dr. Heribert Hofer, for giving me the opportunity to join the spotted hyena project and work on such an interesting topic. Thank you for trusting me in doing this study, for your continuous and stimulating support during the last four years. I have learnt a lot -and not only about hyenas, during our regular meetings and stimulating discussions on my work-whether in the field or in Berlin. Thank you very much for sharing your time, knowledge and british-german sense of humour with me. I am also very thankful to Dr. Bettina Wachter, Eve Davidian and Dr. Oliver Höner, for their friendship, support in the field and for all the discussions and relevant feedback on my manuscripts. Many thanks also to Dagmar Thierer and Kerstin Wilhelm, not only for your impressive efficiency when I needed some information or help, but also for your sense of humour and constant concern.

I am grateful to Prof. Dr. Heribert Hofer and Dr. Christian Voigt for evaluating this present work. I would like to thank all my co-authors, in particular Dr. Stephanie Kramer-Schadt: doing modelling and statistics on R has never been as pleasant as in your company! I have really appreciated your patience, kindness and regular encouragement. I am also very grateful to Dr. Martin Dehnhard for integrating me in the exciting world of endocrinology. Thank you for always taking time to answer my questions, and guiding me when I worked in the lab, and later when writing the manuscripts. I am also grateful to Katrin Paschmionka and Marlies Rohleder; thank you for showing me how to perform ELISA, and for your patience in answering all my questions. Many thanks also to Dr. Wolfgang Goymann from the Max-Planck Institute for Ornithology in Seewiesen, for answering so quickly and precisely to all my questions on hormones, immunoassays and other methodological issues. I also would like to thank Dr. Roberto Bonanni from the University of Parma, for his very helpful comments on the manuscript and always rapid and encouraging feedbacks. I am very grateful to Prof. Dr. Edgar Brunner from the University of Göttingen, for helping me doing a part of our statistical analysis on sibling behaviours- thank you that you immediately accepted to help us and meet me to discuss the analysis. I am also thankful to Prof. Dr. Klaus Eulenberger from the Leipzig Zoo for accepting to conduct the radiometabolism study and for his help on the manuscript, and Martin Weik for collecting the samples. I also would like to thank Janine Helms for providing some of the samples she collected in the field for the hormone analysis. I want to thank Beate Peter-Meyers and Cornelia Greulich for kindly and very efficiently assisting me in the library and in the research of articles. I also thank Silke Ehle, Dagmar Boras, Wolfgang Tauche who helped me for administrative or informatics issues, and who I have always enjoyed meeting somewhere at the IZW. I want to thank Kerstin Austen from the Freie University who kindly and quickly answered my numerous questions on the dissertation, from the enrolment to the submission.

I am grateful to all my colleagues and friends from the IZW/“fg1” for sharing their ideas, discussions, comments on my work- or their kindness. In particular, my little French team: Justine- merci d'être venue vers moi quand je suis arrivée, David, Nelly, Eve, and also Simon, Leif, Mirjam, Friederike, Jan, Katja, Annika, Aines, Pablo, Katharina, Malvina, Martina and Ximena: I am glad I met you on the way.

I am grateful to the Tanzania Commission of Science and Technology, the Tanzania Wildlife Research Institute and the Ngorongoro Conservation Area Authority for their support and for the permission to conduct my research in the Serengeti. I thank Dr. Robert Fyumagwa for his support while in the Serengeti. I am very grateful to all the persons I met in Tanzania, and who offered me their friendship, and helped me while being there. Thomas Shabani: asante sana for your help and continuous presence. Asante sana Berita. Thanks also to Annie Francis and Gideon Orio for helping me in Arusha, to Laura Simpson for her kindness, to Ingela, John and Ayron for being there.

Merci à toute l'équipe du CEFS à Toulouse: en particulier à Dr. Hélène Verheyden- merci pour tout, Dr. Nicolas Morrelet, Dr. Mark Hewison, Bruno Lourtet. Merci à Dr. Dionisios Youlatos de l’Université de Thessalonique pour le très bon encadrement de stage de master!

Finally, I want to thank my dearest family and friends. Georges et Irmelin, Dave et Mike: merci d'avoir supporté sans broncher mon amour immodéré pour les animaux, et ce depuis toujours ! Merci pour tous ces encouragements: vous êtes les meilleurs! Meine liebe Oma, vielen dank dass du immer für mich da warst, und an mich geglaubt hast. Cord, Claas, Ulli: vielen Dank für die wunderschönen Tage in Hamburg, und für die Unterstützung! Vielen Dank an Martin S. Merci beaucoup à mes très chères Annie et Maryse, à Edouard, pour votre sollicitude et votre gentillesse. Merci à ma « deuxième famille »: Ayla, Rifat, Anthony, Cynthia et Linda. Danke Susanne S., daß du mich oft unterstützt hast! Merci ma chère Justine pour mes deux belles premières années à Berlin grâce à toi. Merci David L. pour les grands bols d'air frais bien efficaces! Merci ma Nelly pour les super moments que j'ai pu vivre avec toi, merci Eve pour ton amitié et ta douceur, et ton aide ces deux dernières années! Julie B, merci d'être parmi mes souvenirs les joyeux. Mes chères Sandra et Anne-Claire; merci pour le

soutien efficace, pour nos sorties terribles et délires immortels! Merci ma belle Sandrine pour la vie douce dans la Krossener Strasse depuis plus d'un an! Merci à mes Julien et Giovanni pour ce bon air chaud du sud, pour les merveilleuses « pauses » ensemble, au bord d'une plage à Barcelone ou ici-même au B ;-) Mes adorables Joan et Yoann...c'est simple: à vos côtés, je suis sur un nuage! Conny, manchmal denke ich dass ich dich in einem anderen Leben vielleicht schon kennengelernt habe…danke für die Kraft, die du mir gegeben hast! Danke Jule und Martin! Merci à toi CH. Merci Billy, pour nos moments hors du temps. Merci à ma Marion T, ma meilleure copine depuis 20 ans déjà. Ma très chère Alexa, mon très cher Pierre: comment aurais-je fait sans vous? Mein Alex, danke für deine Unterstützung, dass du mein Leben so lustig und so schön auf w7 in den letzten 2 Jahren gemacht hast. HDSL!

Finally, I am thankful to the IZW for funding my research.

ZUSAMMENFASSUNG

Sowohl Altruismus als auch Egoismus können in Familienverbänden auftreten. Die Rivalität zwischen Geschwistern eines gleichen Wurfes kann intensiv sein und zur Ausbildung einer Dominanzbeziehung führen. Dominante Geschwister erhalten oft einen größeren Anteil der Nahrung, die von den Eltern bereitgestellt wird, und damit enorme Fitness-Vorteile in Bezug auf Wachstum und Überleben. Wenn die durch die Eltern gelieferte Nahrung nicht ausreicht um einen ganzen Wurf erfolgreich aufzuziehen, kann es passieren, dass dominante Geschwister ihre untergeordneten Geschwister töten, entweder direkt durch physische Schäden oder indirekt durch erzwungenen Hunger. Dieses Phänomen wird als "fakultative Brutreduktion" bezeichnet und tritt in einer Vielzahl von Vogelarten und mindestens einer Säugetierart - der Tüpfelhyäne (*Crocuta crocuta*) – auf.

Diese Doktorarbeit wurde durchgeführt, um ein umfassenderes Bild von den Verhaltensmechanismen (Kapitel 2) und den endokrinen (Stress-) Reaktionen auf die Geschwisterrivalität in Zwillingswürfen bei der Tüpfelhyäne (Kapitel 3 und 4) zu erstellen. Die vorliegende Arbeit ist eingebettet in ein Langzeitforschungsprojekt im Serengeti National Park, in Tansania. Dadurch konnten auf umfassende Daten zur Lebensgeschichte mehrerer hundert individuell bekannter Tiere zurückgegriffen werden. In den untersuchten Langzeit-Studiengruppen verstärkt sich die Aggression von dominanten Geschwistern, wenn die Menge der Muttermilch, die für einen Zwillingswurf bereitgestellt ist, sinkt, und fakultative Brutreduktion tritt bei etwa 9.6% der Würfe auf. In **Kapitel 2** habe ich Geschwisterrivalität in einem dynamischen Rahmen von Wechselwirkungen zwischen Dominanten und untergeordneten Geschwistern studiert. In fast allen Würfen versperren dominante Geschwister den unterwürfigen den Zugang zur Muttermilch zu ihren Gunsten, jedoch konnten hungrige und weniger unterwürfige Geschwister diesen Nachteil zugunsten der Dominanten verringern. Das Ausmaß der ungleichen Nahrungsverteilung der Nahrung zugunsten der Dominanten hing von der Wechselwirkung zwischen Wurfalter, Hunger und Geschlecht der beiden Individuen eines Zwillingswurfes ab. Ich liefere überzeugende Beweise, dass weibliche Geschwister stärkere Konkurrenten als männliche sind- und deswegen eventuell ein höheres Potential haben, ihre Geschwister zu töten. Das erklärt höchstwahrscheinlich warum „gefährdetere" unterwürfige Geschwister mit einer dominanten Schwester mehr durchsetzungsfähig sind, als die hungrigen unterwürfigen Geschwister, die mit einem dominanten Bruder aufgezogen werden. Eine Umkehr der Dominanz trat bei 7.0% der Zwillingswürfe auf. Geschulte „Gewinner und Verlierer Effekte" waren bei der Entstehung von Dominanzbeziehungen zwischen dominanten und untergeordneten Geschwistern beteiligt. Eine wichtige Erkenntnis aus diesem Kapitel ist, dass die Dominanten nicht die

absolute Kontrolle über den Zugang ihrer Geschwister zur Muttermilch ausüben, so wie es in Modellen zur Geschwisterrivalität bei Vögeln angenommen wird.

Die Konzentration von Glukokortikoiden wird gewöhnlich als ein Maß für den physiologischen Stress bei Wirbeltieren verwendet. In **Kapitel 3** beschreibe ich ausführlich die Entwicklung und Validierung einer nicht-invasiven Methode zur Messung von Glukokortikoid-Konzentrationen in Tüpfelhyänen-Kotproben. Dafür benutzte ich ein Enzym-Immunoassay, den Cortisol-3-CMO, um fäkale Glukokortikoidmetabolite (fGCM) von Tüpfelhyänen nachzuweisen. Die Validierung dieser Methode umfasste mehrere Schritte. Der Immunoassay dokumentierte erfolgreich erhöhte Konzentrationen von fGCM nach der experimentellen Zuführung von Adrenocorticotropen Hormon (ACTH) bei zwei Tüpfelhyänen in und nach der Anästhesie bei einer weiteren Tüpfelhyäne in menschlicher Obhut. Eine Radiometabolismus-Studie erlaubte es, die fGCM genauer zu charakterisieren. Diese Studie zeigte, dass der Immunoassay vorwiegend Metaboliten mit einer Polarität höher als Cortisol und Metaboliten, die zwischen den Positionen von Cortisol und Corticosteron eluierten, nachweist. Schließlich habe ich diesen Immunoassay benutzt, um paarweise Vergleiche von fGCM Konzentrationen an juvenilen Tüpfelhyänen in der Serengeti durchzuführen. Dabei erwartete ich, dass Jungtiere, die eine weniger vorhersagbare Umwelt erleben, mehr physiologischen Stress zeigen, als ältere Jugendliche. Ich stellte fest, dass weniger als 6 Monate alte Jungtiere höhere Konzentrationen von fGCM hatten als die gleichen Jungtiere im Alter zwischen 6 und 24 Monaten. Dieses Kapitel zeigt, dass der Cortisol-3-CMO-Assay verwendet werden kann, um physiologischen Stress bei Tüpfelhyänen zu messen.

Soziale Unterwerfung und Nahrungsentzug führen oft zu erhöhten physiologischen Stress. In **Kapitel 4** verwende ich die Methode, die ich in Kapitel 3 entwickelt habe, um die Verbindung zwischen Geschwisterrivalität, Geschwistergeschlecht und fGCM in Zwillingwürfen von Tüpfelhyänen zu untersuchen. Vor allem habe ich geprüft, welcher soziale Status im Wurf (dominant oder untergeordnet) den stärksten physiologischen Stress hervorrief. In Übereinstimmung mit meiner Vorhersage fand ich, dass junge subdominante Individuen höhere fGCM als junge dominante aufwiesen, wahrscheinlich, weil subdominante weniger Zugang zur Milch und somit mehr Hunger und Frustration erlebten und weil sie in der Rolle der Unterwerfung „trainiert“ waren. Wie erwartet fand ich auch, dass dominante Weibchen mehr Stress als dominante Männchen erlebten, wahrscheinlich, weil sie mit durchsetzungsfähigeren subdominanten Geschwistern konfrontiert wurden als dominante Männchen (Kapitel 2).

Dies ist die erste Langzeitstudie, die sowohl Daten zum Verhalten als auch zur Endokrinologie bei einer Säugerspezies betrachtet, um im Detail den Zusammenhang zwischen Verhalten und physiologischen Reaktionen bei intensiver Geschwisterrivalität zu erforschen.

SUMMARY

Both altruism and selfishness may occur within families. Rivalry between siblings of a same litter can be intense and lead to a dominance relationship. Dominant siblings often obtain a greater share of the food provided by the parents, and thereby major fitness benefits in terms of growth rate and survival. When food resources provided by the parents are insufficient to rear a whole litter successfully, dominants may kill their subordinate siblings, either directly by physical damage, or indirectly through enforced starvation. This phenomenon is termed “facultative siblicide” and occurs in a wide range of bird species and at least one mammalian species: the spotted hyena (*Crocuta crocuta*).

This thesis was designed to provide a more comprehensive picture of the behavioural mechanisms (chapter 2) and the endocrine (stress) responses to sibling rivalry in twin litters of the spotted hyena (chapter 3 and 4). It was embedded in a long-term research project in the Serengeti National Park, in Tanzania, thanks to which data on life history parameters were available for more than several hundred individually known animals. In the long-term study groups, aggression by dominant siblings intensifies when the amount of maternal milk provided to a twin litter declines and facultative siblicide occurs in approximately 9.6% of the litters. In **chapter 2**, I studied sibling rivalry in a dynamic framework of interactions between dominant and subordinate siblings. Dominants in almost all litters skewed access to maternal milk in their favour, but hungry and less submissive subordinates were able to decrease the skew in favour of dominants. The skew achieved by dominants depended on the interplay of litter age, hunger and sexes of both members of a twin litter. I found compelling evidence that females are better competitors- and hence potentially more likely to commit siblicide - than males. This probably explains why more “at risk” hungry subordinates raised with a dominant sister were more assertive than hungry subordinates raised with a dominant brother. Dominance reversals occurred in 7.0 % of twin litters. Trained “winner and loser effects” were involved in the emergence of the dominance relationship between dominants and subordinates. A key finding of this chapter is that dominants do not exert absolute control over their siblings’ access to milk, as it is assumed in avian models of sibling rivalry.

Glucocorticoids are commonly used as a measure of physiological stress in vertebrates. In **chapter 3**, I detail the development and validation of a non-invasive method for the measurement of glucocorticoid concentrations in spotted hyenas. For this purpose I used an enzyme-immunoassay, the cortisol-3-CMO, to detect faecal glucocorticoid metabolites (fGCM) of spotted hyenas. The validation of this method comprised several steps. The assay success-

fully measured increased concentrations of fGCM after adrenocorticotropic (ACTH) hormone challenges in two captive spotted hyenas, and after anaesthesia of another captive spotted hyena. In addition, a radiometabolism study was used to characterise fGCM, and showed that the assay mostly detected metabolites with a polarity higher than cortisol and metabolites that eluted between the positions of cortisol and corticosterone. Finally, I used this assay to conduct pairwise comparisons of fGCM concentrations in Serengeti juvenile spotted hyenas when less than 6 months of age and when between 6 and 24 months of age. I expected juveniles to experience a more unpredictable and therefore more stressful environment when younger than when older, and found that young juveniles indeed had significantly higher fGCM concentrations than when they were older. This chapter demonstrates that the cortisol-3-CMO assay can be used to measure physiological stress in spotted hyenas.

Social subordination and food deprivation often lead to increased physiological stress levels. In **chapter 4**, I used the method developed in chapter 3 to investigate the link between sibling rivalry, sibling sex and fGCM in spotted hyena twin litters. More specifically I tested which within-litter status (i.e. dominant vs. subordinate) carried the most physiological stress. As expected, I found that young subordinates had higher fGCM concentrations than young dominants, probably because subordinates have less access to milk, experience more hunger and frustration, and are trained into submission. I also found that dominant females were more stressed than dominant males, probably because they were confronted by unpredictable, assertive siblings, whereas dominant males were not (chapter 2).

This is the first study that combines behavioural and endocrine data from a long-term study on a mammalian species, to explore in detail the link between behavioural and physiological responses to intense sibling rivalry.

CHAPTER 1: GENERAL INTRODUCTION

Although family members provide each other with indirect fitness benefits through inclusive fitness, intra-family conflicts both between parents and offspring and between siblings may occur (Mock and Parker 1997). Hamilton’s rule on altruism (1964) does not only predict the conditions under which individuals should behave altruistically on behalf of a genetic relative, but also the conditions under which selfish behaviours are expected to emerge among close kin. Sibling rivalry is broadly defined as “any features of animals or plants that have the effect of promoting individual survival and / or reproduction at the expense of siblings” (Mock and Parker 1997). Theoretically, siblings are expected to avoid escalated conflicts unless the fitness benefits provided outweigh the fitness costs that arise from them (Parker et al. 1989, Godfray and Harper 1990). It has been shown in many bird species that siblings that skew food resources provided by parents in their favour obtain fitness benefits (Mock and Parker 1997). The majority of empirical and theoretical research on sibling rivalry has been conducted on birds where dominant siblings seek to out-compete their broodmates for food provided by parents, either by the higher intensity of begging or by aggression directed towards their broodmate(s) (or both).

Rivalry between members of a brood or litter may lead to the emergence of a “teat order” (e.g. in domestic pigs (*Sus scrofa domestica*; Fraser and Thompson 1991)) or a dominance relationship (Drummond 2006). In those species in which offspring can influence the allocation of food delivered by parents, the dominant sibling is expected to seek to skew parental food provisioning in its favour (Fraser and Thompson 1991, Drummond and Osorno 1992, Hofer and East 2008) and the degree of skew in its favour is expected to increase as parental provisioning declines (Fujioka 1985, Ploger and Mock 1986, Parker et al. 1989, Godfray and Harper 1990, Forbes and Ydenberg 1992, Mock and Parker 1997). Thus by out-competing their siblings, dominants obtain the fitness related benefits of increased growth and survival, whereas subordinate littermates can suffer reduced growth rates and survival. These effects of dominance relationships within litters have been reported in several bird species, including the great blue heron (*Ardea herodias*), the great egret (*Ardea alba*) and the cattle egret (*Bubulcus ibis*) (Mock and Parker 1986, 1997), the western gull (*Larus occidentalis*; Spear and Nur 1994) and the blue-footed booby (*Sula nebouxi*; Guerra and Drummond 1995, Osorno and Drummond 1995), and in one mammalian species, the spotted hyena (*Crocuta crocuta*; Hofer and East 2008).

When sibling rivalry is intense it can eventually lead to the death of the subordinate member(s) of a brood or litter through enforced starvation, physical damage or eviction from the nest (Mock and Parker 1997). This fatal sibling competition is termed siblicide (or brood reduction) and is well documented in birds, where this phenomenon is common, particularly in raptors such as the osprey (*Pandion haliaetus*; Machmer and Ydenberg 1998), or in colonially nesting birds such as blue-footed boobies (Drummond and Garcia Chavelas 1989), brown boobies (*Sula leucogaster*; Drummond et al. 2003), great blue herons or kittiwakes (*Rissa tridactyla*; reviewed by Drummond 2006). In contrast, siblicide has been reported in only a few mammalian species, including the spotted hyena (Hofer and East 1997, 2008), the Galápagos fur seal (*Arctocephalus galapagoensis*; Trillmich and Wolf 2008), possibly the Arctic fox (*Alopex lagopus*; Macpherson 1969) the red fox (*Vulpes vulpes*; Henry 1985) and the Indian false vampire bat (*Megaderma lyra*; Leippert et al. 2000). Siblicide may either be "facultative" when it is adjusted to shortages in the environment that lead to a decrease in the level of food provided by parents, or it is "obligate" when the lowest ranking sibling is routinely killed by its dominant broodmate or littermate. Within-litter dominance status may therefore substantially affect an offspring's current and future fitness (Höner et al 2010), and investigation of the factors influencing the emergence of dominance relationships among siblings has received considerable attention in birds.

1.1 Initial competitive asymmetries in birds

1.1.1 Hatching asynchrony

In most siblicidal bird species, eggs hatch asynchronously. Hatching intervals between successive eggs can be large, e.g. up to five or six days between the first and second chick in the two-chick broods of the brown booby and the Nazca booby (*Sula granti*; Mock and Parker 1997). Asynchronous hatching has been previously explained as an adaptive mechanism by parents to facilitate siblicide in habitats with unpredictable food resources (Lack 1954). The first-hatched chick has several days in which it is fed and thus has an initial competitive advantage over its sibling, perhaps because it has a higher body size or body mass (as suggested by Werschkul and Jackson 1979, Bortolli 1986, Machmer and Ydenberg 1998, and Groothuis et al. 2005), or because it has a better fighting ability in terms of age, speed and motor coordination (Mock and Parker 1997) when the junior sibling finally hatches. Thus, first-born chicks emerge as dominants in facultative or obligate siblicidal bird species with hatching asynchrony (Mock and Parker 1997, Drummond et al. 2001, 2006). During feeding events, dominant chicks may monopolise a position close to the parent and use aggression when a subordinate sibling persists in approaching the parent, begs loudly or contests access to food (Machmer and Ydenberg 1998). Aggression by dominant chicks often causes the subordinate chick to hesitate at the critical moment of a food item delivery (Mock 1985, Fujioka 1986, Ploger and Mock 1986). Subordinate chicks are thus usually fed only once (if ever) the senior chick is satiated, and may not be fed at all if the number of food items delivered by the parents is insufficient for the entire brood (Inoue 1985, Mock 1985, Ploger and Mock 1986).

1.1.2 Maternal androgens

Differential maternal investment in egg "quality" (e.g. size, mass, carotenoids or antibody levels) or androgens deposited in egg yolks may have a strong impact on the phenotype of offspring (Groothuis et al. 2005), and hence on the development of within-brood competitive asymmetries. Egg quality may affect early growth and survival of chicks in some species, for example larger eggs give rise to heavier chicks at hatching (reviewed by Williams 1994, also see e.g. Blount et al. 2002). Elevated levels of androgen in egg yolks generally improve growth, begging behaviour or early survival of chicks before fledging in several species (reviewed by Groothuis et al. 2005). For example, in captive canaries (*Serinus canaria*), mothers deposit higher titres of testosterone in the yolks of last-laid eggs, thereby enhancing the aggressiveness of these chicks towards their broodmates (Schwabl 1993). The pattern of increased testosterone with laying order is thought to compensate the disadvantage of being a later-hatched chick in non-siblicidal species (Groothuis et al. 2005) and has also been detected in other species such as the black-headed gull (*Larus ridibundus*; Eising et al. 2001) and the lesser black-backed gull (*Larus fuscus*; Verboven et al. 2003).

Alternatively, the level of maternal androgens in yolk can decrease with the laying sequence, as in the cattle egret (Schwabl et al. 1997) and possibly the white stork (*Ciconia ciconia*; Sasvari et al. 1999). In both facultative siblicidal species, higher androgen levels in the first egg seem to work in concert with hatching asynchrony to help the senior chick to discard its subordinate sibling when food shortages occur (Schwabl et al. 1997). Nevertheless, a recent study revealed that in blue-footed and brown boobies, two other facultative siblicidal species, contrary to expectation, the first, second and third eggs showed no difference in levels of 5α-dihydrotestosterone, testosterone and androstenedione (Drummond et al. 2008). In the blue-footed booby, but not the brown booby, the absolute yolk masses declined with laying order. In these species, initial competitive asymmetries between broodmates are thus mainly created through a marked hatching asynchrony, and in the blue-footed booby through asymmetry in yolk mass (Drummond et al. 2008), but probably not through variability of maternal androgens deposited in yolk. Finally, an example in contrast to all these species is the American kestrel (*Falco sparverius*) where higher concentrations of these hormones decrease chick survival (Sockman and Schwabl 2000). In conclusion, the link between siblicide, hatching asynchrony, egg quality and the pattern of yolk androgen level deposition over the laying sequence is still under debate (see Groothuis et al. 2005 for a discussion).

1.1.2 Sexual dimorphism

Sex differences in competitive abilities may also affect the dynamics of sibling rivalry. In particular, sexually dimorphic growth could confer a competitive advantage on siblings of the larger sex (Lack 1954). Patterns of sexual dimorphism vary among bird species, with males being much larger than females in some species such as rooks (*Corvus frugilegus*) and new world blackbirds (Mock and Parker 1997) or great tits (*Parus major*; Oddie 2000), and females being much larger than males in others, as in many raptors (Bortolotti 1986) and owls (Mock and Parker 1997). In the highly dimorphic brown songlark (*Cinclorhamphus cruralis*), females hatch from larger eggs and are initially heavier than males (Magrath et al. 2003). Although the effect of such dimorphism on sibling rivalry has not yet been demonstrated in this species, it may provide females with a competitive advantage against brothers. In the red-winged blackbird (*Agelaius phoeniceus*), males are larger than females and male siblings do efficiently use their height advantage to intercept food delivered by the parents (Teather 1992). In American kestrel chicks, the larger body size of females, from shortly after hatching through fledging, provides female siblings with a competitive advantage because they managed to monopolise more food items during scramble competition than their smaller brothers (Anderson et al. 1993). In bird species in which sibling rivalry for food is influenced by chick size, sexual size dimorphism could result in a sex-biased mortality, as suggested by results found in broods of the bald eagle (*Haliaeetus leucocephalus*), a siblicidal species (Bortolotti 1986).

However, the hypothesis that in sexually dimorphic bird species the smaller sex must necessarily show a higher mortality rate as a result of its size disadvantage during conflicts, has been contradicted by the experimental results of Drummond et al. (1991) in blue-footed boobies. Although dominant males were systematically outgrown by their subordinate sisters by their 40th day of life (subordinate females become 27% heavier than their dominant males at this age), they were never out-competed by their larger sisters. This result has later been interpreted (Drummond and Osorno 1992, Drummond and Canales 1998) as being the consequence of early social experience which may have a more robust effect in itself than relative size (mass) or age differences between broodmates (discussed in the next section).

1.2 Trained winner and loser effects

Trained winner and loser effects are the results of learning processes in that the experience of winning or losing a contest alters an individual's probability of winning or losing contests with future competitors (Chase et al. 1994, Drummond and Canales 1998, Chase et al. 2002), possibly because these experiences shape the assessment of an individual's own fighting ability (Hsu et al. 2006). The properties of winner and loser effects have been investigated using theoretical models (e.g., van Doorn et al. 2003, Fawcett and Johnstone 2010). Evidence for their existence was experimentally demonstrated in adults in several vertebrate (mice, birds, fishes, Chase et al. 1994) and invertebrate species (e.g. insects, see Hsu et al. 2006 for a review).

The dominance of blue-footed booby brothers over their heavier subordinate sisters (Drummond et al. 1991) led to the first suggestion that relative sibling size (mass) may not be the ultimate and only determinant of rivalry and dominance in species with facultative siblicide. Instead, in this species the basis of the dominance relationship is also a function of social experience, i.e., the tendency to behave aggressively or submissively (Drummond and Osorno 1992), later identified as trained winner and loser effects (Drummond and Canales 1998). In the blue-footed booby, the four days older, first-hatched chick trains its subordinate sibling to become a "loser" during the first days or weeks of life (Drummond 2006). As a consequence, trained subordinate chicks learn to reply with submission to threats by dominants throughout the entire nestling period (Drummond 2006). The relative contributions of the differences in size (mass, age) versus training effects on the emergence of the dominance relationship were assessed by experimental manipulations in blue-footed boobies (Drummond and Osorno 1992). In chicks lacking social prior experience with a sibling (i.e. singletons), the relative size (mass) or age of broodmates determined the direction of dominance, since in almost every pair, the larger, heavier and older chick became the dominant. But do asymmetries in such attributes prevail over the effect of trained winner and loser effects? To answer this question, the authors formed experimental pairs with a dominant and a subordinate that was on average 32% heavier and 4 days older. As a control, they used pairs where chicks were similarly fostered between nests but the natural size and age asymmetry of dominant and subordinate broodmates were maintained. Although heavier subordinate siblings paired with smaller dominants were more aggressive than controls in the first few days after the experimental pairing, their aggression then strongly decreased, and only one (out of 12) of these larger subordinates achieved dominance, suggesting that the effect of experience (i.e., previous training as a loser or winner) eventually prevailed over the effect of asymmetries in size (mass) or age. This also provided an explanation for the fact that heavier subordinate females did not outcompete their older brothers (see above, Drummond et al. 1991). Trained winner and loser effects may be involved in the emergence of a dominance relationship between broodmates or littermates in other species than the blue-footed booby, as suggested by Drummond (2006).

1.3 Behavioural responses of subordinate siblings

While many studies have explored the factors influencing variation in the intensity of aggression by dominants towards subordinate siblings (e.g. Mock et al. 1987, Machmer and Ydenberg 1998, Golla et al. 1999, Cook and al. 2000, Wachter et al. 2002, Wahaj and Holekamp 2006), considerably less attention has been given to the behavioural responses of subordinate siblings to this aggression (but see Drummond 2006 for anecdotal evidence in several species). This may be because aggressive behaviours are more obvious and easier to measure than submissive responses to aggression. However, behavioural responses of subordinate siblings to aggression by dominants could have an important influence on the dynamics and expression of sibling rivalry (Drummond 2006).

For instance, hunger may constitute a strong motivation in itself to win conflicts and achieve dominance, as it has been demonstrated in an elegant experimental manipulation by Rodrigues-Girones et al. (1996) in the blue-footed booby. Unrelated subordinate chicks with different levels of hunger but of similar age (to maintain similar fighting abilities between contestants) were paired. Hungrier subordinates always became dominant, despite having to compete against broodmates of similar age which happened to be 35% heavier (in one brood). This result is consistent with game-theoretical models which assume that a contestant that would benefit from a resource more than the other, should be more eager to fight and more likely to obtain it, unless the asymmetry in fighting abilities is too large (Hammerstein 1981, Einquist and Leimar 1987). In natural broods, an asymmetry in training effects (i.e. a trained winner sibling against a trained loser sibling) may similarly prevent or at least decrease the ability of hungry subordinates to win a contest and obtain a valuable resource (i.e. food or dominance status). According to Rodrigues-Girones et al. (1996), assertiveness of hungrier subordinate siblings should not lead to dominance reversals in a majority of natural broods because before reversals happen, the dominant chick would become food-deprived itself, and the value it assigns to dominance would increase (Rodrigues-Girones et al. 1996). In addition, dominant siblings are expected to remain better competitors than subordinates throughout at least the entire infancy, because they have an initial age or size advantage compared to their siblings and / or because they are trained winners. However, rare cases of dominance reversals have been reported in some species (Drummond 2006).

In species showing obligate siblicide such as in the brown booby, subordinate chicks usually lack submission and instead show intense “desperado behaviours”, i.e., aggression towards dominant chicks. The “desperado sibling hypothesis” (Drummond et al. 2003) derives from the game theoretical model of the “desperado effect” (Grafen 1987) and describes the extreme aggressiveness of subordinate chicks that have no survival prospects in obligatory siblicidal species.

Among broods or litters of facultatively siblicidal species, subordinates are expected to facultatively adjust their level of submission according to their survival prospects, i.e., to decrease their level of submission towards dominants as the cost of subordination in terms of likelihood of starvation increases (Drummond 2006). Hence, if parents bring enough food for the entire brood to survive to independence, then subordinates are supposed to accept their inferior status and show submission in response to aggression by dominants (Drummond 2006). In contrast, when the amount of food provided by the parents is insufficient for the entire brood to survive, then subordinates are expected to decrease their level of submission. Indeed, as complete acceptance of subordination by hungry chicks would result in their death, selection should act against such behaviour (Rodrigues-Girones et al. 1996). Empirical evidence suggests that hungrier subordinates are more likely to show assertive or aggressive behaviour towards their dominant siblings in some species such as the great egret (*Egretta alba*; Mock and Parker 1997), the brown pelican (*Pelecanus occidentalis*; Pinson and Drummond 1993) or the black guillemot (*Cepphus grylle*; Cook et al. 2000). It is unknown whether these behavioural responses by subordinates increase their share of food resources.

Finally, sibling competitive asymmetries established through (1) hatching asynchrony, (2) differential maternal investment in egg quality and androgens, or (3) winner and loser effects theoretically provide the senior chick with a relatively higher competitiveness than that of the younger chick(s) (Parker 1974, Hammerstein 1981, Enquist and Leimar 1987). This results in an asymmetric rivalry which enables the senior chick to commit siblicide when food resources provided by the parents are below the threshold required to raise the entire brood (Parker et al. 1989). Theoretical models of siblicide assume that the within-brood dominance order is rigid and that senior chicks exert absolute control over their subordinate siblings and thereby can monopolise parental resources until they are satiated (Parker et al. 1989, Mock and Parker 1997). Field studies are broadly and qualitatively consistent with these models in many bird species in which senior chicks use facultative aggression to efficiently increase their share of food (e.g. Forbes 1989, and Mock 1986, Machmer and Ydenberg 1998), and in which dominance relationships appear stable over time and reversals rare (Drummond et al. 1991).

1.4 Sibling rivalry in mammalian species

Bird studies still dominate the sibling rivalry literature and have stimulated most theoretical models (e.g., Parker et al. 1989, Mock et al. 1990 but see Stockley and Parker 2002), perhaps because the experimental manipulation of eggs and young birds is easier to achieve in practice than is the manipulation of foetuses or young in many mammalian species. In mammalian species, most studies focused on mother-offspring conflicts, and studies dedicated to the influence of sibling interactions on offspring development are rare (Hudson and Trillmich 2008). Currently, there is no evidence of obligate siblicide in any mammalian species, and in contrast to many bird species, mammalian mothers give birth to littermates almost simultaneously, often within minutes or hours. Through lactation, sucking mammalian offspring probably experience a very different feeding situation than bird chicks. Indeed, mothers possess several teats or nipples and nurse their offspring simultaneously (Hudson and Trillmich 2008). During suckling bouts, a mother usually adopts an immobile posture, typically standing over a litter as in rodents or ungulates, or lying on the side as in pigs or carnivores (Hudson and Trillmich 2008), and hence might have fewer options than a parent bird to favour (or disadvantage) a specific sibling (but see the study by Trillmich and Wolf 2008 in the Galápagos fur seal). In addition, as milk ejection occurs simultaneously at all teats, offspring theoretically are expected to end agonistic interactions over obtaining milk as soon as it becomes available (Hudson and Trillmich 2008). It is worth considering whether the predictions that derive from the models developed for sibling rivalry in birds apply to rivalry in the biologically rather different mammalian system. In particular, the behavioural processes involved in the emergence of dominance relationships and the tactics of competing siblings remain unclear in mammalian litters.

1.5 Effect of sibling rivalry on physiological stress

While most studies have focused on the behavioural aspects of sibling rivalry, few investigations were directed to the physiological processes linked to sibling rivalry, in particular the physiological stress responses to intense sibling rivalry (but see Nuňez-de-la-Mora et al. 1996, Fey and Trillmich 2008). Frequent aggression combined with food deprivation is likely to be stressful and stimulate the adrenocortical activity of young animals showing intense sibling rivalry. Vertebrates respond to perceived stressors through increased plasma concentrations of glucocorticoids (e.g. cortisol, corticosterone). These steroid hormones are released by the adrenal cortex in the bloodstream after activation of the hypothalamic-pituitary-adrenal (HPA) axis (Goymann and Wingfield 2004).

Glucocorticoids may have opposing effects upon health and individual fitness: their short-term release helps vertebrates to overcome potentially dangerous situations through the rapid mobilisation of energy (see Romero et al. 2009), but chronically elevated levels of glucocorticoids ("chronic stress") may have important deleterious effects on growth (Wada 2008), on cognition (Mateo 2008), on the immune system (Munck et al. 1984), on reproductive performance (Liprap 1993, Ferin 1999), and possibly on survival (Pride 2005). Over the last 15 years, the measurement of glucocorticoids has been increasingly used to assess the health of free-ranging populations (Wasser et al. 1997, Romero 2004), or to investigate the relationship between hormone concentrations and behaviour, for instance the link between social dominance and stress hormones (Abbot et al. 2003, Goymann and Wingfield 2004, Sapolsky 2005).

Glucocorticoids have traditionally been measured in blood plasma, but capture or anaesthesia of animals to obtain samples are stressful and often lead to significant increases in glucocorticoid concentrations (Sapolsky 1982, Wingfield et al. 1994). Furthermore, plasma glucocorticoids are only representative for a narrow time frame because their secretion may fluctuate on a circadian basis (Monfort et al. 1993). Circulating steroid hormones are usually metabolised by the liver and excreted as conjugates via the kidneys into the urine or via the bile into the gut (Palme et al. 1996). Steroid metabolites can be detected in the faeces of mammalian species or in bird droppings, and alternative non-invasive methods to assess faecal glucocorticoid metabolite (fGCM) concentrations, as they do not induce stress, are preferable to investigate the factors influencing adrenocortical activity, especially in free-ranging animals. These methods are based on hormone assays such as enzyme immunoassays (EIAs) (reviewed most recently by Hofer and East 2012) and are increasingly used in the field of behavioural endocrinology to investigate the effect of environmental, seasonal (e.g. Wingfield et al. 1994) or social (Goymann et al. 2001a) stressors on wildlife.

However, determination of fGCMs may not be straightforward. Indeed, significant differences regarding the metabolism and excretion of glucocorticoid metabolites can occur between even closely related species or within a same species or between the sexes (Touma and Palme 2005). In addition, there is considerable variation in the time lag between an increase in glucocorticoid concentration in plasma and the appearance of metabolites in the faeces. This time lag mainly depends on the intestinal transit time from the dueodenum to the rectum and can vary between, and possibly within, species to a great extent (Palme et al. 1996). After defecation, temperature, humidity and bacterial enzymes can influence the concentrations of fGCM (e.g. Khan et al. 2002). For all these reasons, assays used to measure steroid metabolites in faeces should be carefully validated for each vertebrate species to which any given assay is applied, before being used to measure changes in adrenocortical activity in a specific species (Touma and Palme 2005).

Validation typically requires pharmacological stimulation of the HPA axis to evaluate whether stimulation of glucocorticoid production leads to an increase in measured concentrations of fGCMs. The most widely used experiment to stimulate adrenocortical activity is the injection of adrenocorticotropic hormone (ACTH) which activates the secretion of glucocorticoids from the adrenal cortex (Goymann et al. 1999, Touma et Palme 2005). In addition to this physiological validation, providing a biological validation of the assay is a crucial step. To this purpose, fGCM are generally compared before and after a known stressful event such as capture, anaesthesia or translocation (Goymann et al. 1999, reviewed by Touma and Palme 2005).

Despite recent advances in the non-invasive monitoring of adrenocortical activity, these methods have never been used to examine the endocrinology of competing mammalian siblings. Because subordinate littermates may suffer intense hunger, trained loser effects, frustration and / or reduced growth and survival, the experience of being the subordinate member of a twin litter is likely to be more stressful than of being the dominant in most species showing intense sibling rivalry and facultative siblicide. Such an effect was detected in birds, where young subordinate chicks showed higher levels of plasma corticosterone than dominants in the blue-footed booby (Nuňez-de-la-Mora et al. 1996) and in the Nazca booby (Tarlow et al. 2001). Interestingly, when dominant blue-footed booby chicks (which normally experience submission from their subordinate siblings) were experimentally paired with non-submissive singletons, they showed an increase in plasma corticosterone concentrations (Ramos-

Fernandez et al. 2000), indicating that a dominant position can be stressful if strongly contested and challenged by subordinates (reviewed by Goymann and Wingfield 2004).

1.6 Sibling rivalry in spotted hyena twin litters

1.6.1 Background to sibling rivalry

Fig 1: A long dependence on maternal milk: a mother suckles her approximately 13 month old twin litter. Photo: S. Benhaiem.

The spotted hyena is one of the few mammalian species that exhibits intense sibling rivalry that can lead to facultative siblicide (Golla et al. 1999, Smale et al. 1999, Wachter et al. 2002, Hofer and East 1997, 2008). Females give birth to mostly singleton and twin litters, rarely triplets, throughout the year. Spotted hyena neonates exhibit the precocial traits of having their eyes open and teeth (incisors and canines) erupted at birth (Pournelle 1965), and the altricial trait of absolute dependence on highly nutritious maternal milk throughout the initial 6 months of life, although lactation usually lasts a minimum of 12 months (Hofer and East 1993c, 1995, see Fig 1). The precocial traits of spotted hyena cubs probably evolved for use during intense early sibling rivalry for within-litter dominance soon after birth, which provides major fitness benefits in terms of improved growth and survival for dominant littermates (Hofer and East 1997, 2008) and the possible fitness cost of facultative siblicide through enforced starvation for subordinate littermates (Hofer and East 1997, 2008, Golla et al. 1999). Facultative siblicide occurs in 9.6% of litters in the Serengeti National Park, Tanzania (Hofer and East 2008). Both precocial and altricial traits are linked in that early within-litter dominance provides control over access to maternal milk during the exceptionally lengthy lactation period (East and Hofer 2002, Hofer and East 2008).

Fig 2: Serious wounds inflicted on the runt of a triplet litter during sibling conflict in the Serengeti National Park. Photo: S. Benhaiem

In this species, birth was observed in captivity (Frank et al. 1991, Smale et al. 1995) and siblings in twin or triplet litters were reported to be born at an interval of about one hour. According to these studies, the first born cub attacks the second born cub immediately after it emerges and the second born cub quickly fights back. A dominance relationship emerges within a few days (Smale et al. 1995). These observations leave open whether the first born cub typically becomes dominant and the second born cub the subordinate. Sibling rivalry is intense during the first 3-4 months of life (see Fig 2) and then gradually declines as litter age increases (Golla et al. 1999, Drea et al. 1996, Smale et al. 1999). Golla et al. (1999) demonstrated that when twin hyenas competed for access to maternal milk supplies, dominants were three times more aggressive than subordinate siblings.

1.6.2 Monopolisation of maternal teats by dominants

Spotted hyena females possess two functional teats and dominant siblings suckle in a preferred position against the mother's belly. Subordinate siblings suckle from a posterior position between the mother's hind legs and thus have a more restricted access to maternal milk. This configuration enables dominant siblings to monopolise access to both maternal teats should they wish to do so (Fig. 3). In contrast to birds, where chicks compete for each discrete food item brought by parents to the nest (e.g., Drummond and Garcia Chavelas 1989, Mock and Parker 1997, Machmer and Ydenberg 1998, Cook et al. 2000), dominant spotted hyena cubs must terminate their own milk intake to exclude a subordinate and hence aggression can be costly in terms of lost suckling time.

Fig 3. Dominant siblings monopolise the access to both maternal teats. Top: the subordinate attemps to reach a teat, while the dominant suckles. Bottom: the dominant stopped suckling, threatens the subordinate which responds with submission (retreats and ears back). Photos: S. Benhaiem

1.6.3 Effect of sex on sibling rivalry

The level of sibling aggression was reported to vary with the sex composition of the litter, be most intense among same-sex litters and particularly among sisters (Golla et al. 1999, Smale et al. 1995), possibly because females do not disperse and thus sisters directly compete for social status when they integrate into the adult female hierarchy (Hofer and East 1997, Golla et al. 1999). In Serengeti spotted hyenas, the primary sex ratio is not biased (the proportion of males is 0.52; Hofer and East 1997). However, it masks a female bias in singleton litters after the age at which siblicide usually occurs (proportion of males 0.43; Hofer and East 1997, James and Hofer 1999). This suggests that females born in same-sex litters (as modelled by James and Hofer 1999) are very competitive and are more likely to commit siblicide than cubs in litters with any other sex composition. It is therefore conceivable that females may survive sibling rivalry more often than males also in mixed-sex litters (Hofer and East 1997, James and Hofer 1999), and thus may generally be better at monopolising teats than males.

Testosterone produced by the placenta through metabolic conversion of maternal androgens is transferred to spotted hyena foetuses (Licht et al. 1992). However, as males and females experience the same prenatal maternal environment enriched with androgens (in captivity; Licht et al. 1992, Dloniak et al. 2006), and as no differences in androgens between males and females were found during the first month of life (Frank et al. 1991), and later as adults (Goymann et al. 2001b), it seems unlikely that elevated androgen concentrations lead to increased aggressiveness in a particular sex.

1.6.4 The consequences of the Serengeti commuting system on sibling rivalry

Spotted hyena clans are stable multi-female, multi-male fission-fusion groups with separate linear dominance hierarchies among philopatric adult females, and a similar number of reproductively active, mostly immigrant adult males (Hofer and East 1995). All adult females are socially dominant over males. Most males disperse whereas females remain within their natal clan, where they live in a linear dominance hierarchy structured by matrilines. Indeed, in this species offspring “inherit” a social status similar to and immediately below that held by their mothers (Hofer and East 2003, East et al. 2009). Rank inheritance is a consequence of maternal behavioural support of offspring during encounters with other clan members when offspring integrate themselves into the adult dominance hierarchy (East et al. 2009). Daughters keep this social status more or less for their entire life; sons are treated as if they held the same status as their mother until they become reproductively active or emigrate to a new clan (Smale et al. 1993). Benefits linked to social dominance, such as priority of access to food resources, are passed to offspring through generations, a phenomenon termed the “silver spoon effect” (Frank 1986, Hofer and East 1993b, 2003).

In the Serengeti National Park, maternal input to offspring in terms of milk is strongly influenced by the “commuting system”. This phenomenon combines the effects of both the social status of a mother and the fluctuation in prey abundance on maternal nursing rates (Hofer and East 1993a,c). In this ecosystem, the abundance of prey within clan territories (approximately 56 km^2; Hofer and East 1993a) fluctuates greatly, due to the movement of migratory herbivores, typically wildebeests, *Connochaetes taurinus*, Thomson’s gazelles, *Gazella thomsoni*, and zebras, *Equus burchelli* (Hofer and East 1993a,b). Clan territories usually do not contain sufficient prey resources for all clan members (Hofer and East 1993b) and fluctuations in prey abundance profoundly affect mothers' ability to provide dependent cubs with milk. The period of low abundance of prey (total prey density ~ 3.3 animals km^{-2}, Hofer and East 1993a) occurred during approximately 37% of the year in the study clans of this thesis. These unpublished data are based on more than 20 years of observations in the study clans holding territories in the centre of the Serengeti National Park, outside the typical wet and dry season ranges of the migratory herbivores. During this period, only resident herbivores are present such as buffalos (*Syncerus caffer*), warthogs (*Phacochoerus africanus*) and impalas (*Aepyceros melampus*; Hofer and East 1993a) and all migratory herbivores are either absent or present in very low numbers. At this low level of prey abundance, lactating females of all social status leave their dependent offspring at the clan's communal den for several days and forage on distant (up to 70km from their territory), high concentrations of migratory prey ("commuting trips", Hofer and East 1993b,c).

The period of medium abundance of prey occurs during approximately 50% of the year in the study clans. During this period, resident herbivores and migratory herbivores are present in moderate numbers (total prey density ~31 animals km^{-2}; Hofer and East 1993a), and all high- and many mid-ranking female hyenas typically forage within their territory, whereas all low-ranking females conduct long-distance foraging trips (Hofer and East 2003).

The period of high abundance of prey occurs approximately 13% of the year in the study clans and is characterised by a super-abundance of migratory herbivores plus resident herbivores (total prey density ~238 animals km^{-2}; Hofer and East 1993a). All female hyenas forage inside the territory and nurse their offspring daily, irrespective of social status (Hofer and East 1993c).

Changes between levels of prey abundance occur repeatedly throughout the year, thereby influencing the extent of long-distance foraging and hence the level of maternal input to offspring (Hofer and East 1993b,c, 1997, 2003, 2008). During these absence intervals, cubs less than six months of age remain within the communal den and are thus likely to experience long intervals (up to seven days) between visits by their mother at the communal den to nurse them (Hofer and East 1993c). As the interval between nursing visits of a mother decreases with increasing social status, offspring of dominant females are better fed than those of subordinate females and as a consequence, exhibit higher growth and survival rates (Hofer and East 2003, 2008). The overall rate of sibling aggression increases as the interval between nursing visits by the mother increases, but also as maternal social status declines (Golla et al. 1999). Although sibling rivalry in spotted hyena litters has been reported in a diverse range of habitats (e.g. Kalahari desert in southern Africa; Mills 1990, Masai Mara National Reserve in Kenya; Smale et al. 1999, Ngorongoro Crater, Tanzania; Wachter et al. 2002, in captivity; Smale et al. 1995), facultative siblicide very rarely occurs in habitats with relatively high and stable densities of resident prey (e.g. in the Masai Mara National Reserve; Smale et al. 1999; Ngorongoro Crater; Wachter et al. 2002) or in captivity where food is abundant (Frank et al. 1991).

1.7 Objectives of this thesis

A fundamental condition for the evolution of facultative siblicide is unpredictability of food resources which ultimately influences offspring growth and survival through within-brood or litter rivalry (Mock and Parker 1997). The intensity of sibling rivalry and the conditions leading to facultative siblicide in the spotted hyena have previously been the focus of attention (Golla et al. 1999, Smale et a. 1995, Drea et al. 1996, Wachter et al. 2002, Hofer and East 1997, Wahaj and Holekamp 2006, Hofer and East 2008).

The first aim of this thesis was to assess whether the main assumptions that derive from the "bird models" of sibling rivalry apply to rivalry between spotted hyena siblings. I first focused on the tactics used by both siblings to obtain exclusive access to maternal milk during rivalry. I addressed the neglected role of the subordinate sibling to assess whether dominants exert absolute control over their littermates' access to maternal teats during conflicts. In birds, dominant siblings are assumed to have absolute control over their subordinate broodmates' access to food, because they become more aggressive during food shortages and obtain a disproportionate share of the food provided by the parents. Absolute control was assumed by models of facultative siblicide based on bird species (e.g. Mock et al. 1987, Parker et al. 1989), and an increase in aggression and a disproportionate share of the dominant demonstrated using experimental manipulation (Drummond and Garcia Chavelas 1989, Machmer and Ydenberg 1998, Cook et al. 2000) and observed in natural populations (e.g., Fujioka 1985, Ploger and Mock 1986, Drummond et al. 1986). In Serengeti spotted hyena twin litters, dominant siblings should seek to exert a high level of behavioural control over access to maternal teats as this would ensure absolute priority of access to milk during the exceptionally long lactation period, and the ability to discard their siblings when milk provided by the mother is insufficient for the entire litter to survive. This behavioural control may be achieved through training effects during repeated encounters. In this mammalian society with female philopatry and female social dominance, I also tested whether females have a competitive advantage against males during conflicts.

The second purpose of this thesis was to investigate whether sibling rivalry induces stress in competing littermates, first by validating a new non-invasive method for the measurement of glucocorticoids in the faeces of spotted hyenas, and second by using this method to link the behaviour of competing siblings with their level of glucocorticoids. Increased levels of glucocorticoids may result from food deprivation (Nuňez-de-la-Mora et al. 1996) or competition for food (Fey and Trillmich 2008). As subordinates in twin litters are more likely to suffer elevated hunger, intense sibling rivalry in spotted hyena litters is most likely to result in higher concentrations of glucocorticoids in the subordinate sibling. However if maintenance of the position of dominance requires a high cost (Goymann and Wingfield 2004), i.e., if subordinates show assertive behaviour and behaviourally challenge dominants, then dominants should show higher levels of physiological stress.

The results of my studies are presented in three manuscripts in chapters 2 to 4:

Chapter 2: Investigates the behavioural factors that influence the degree of control exerted by dominants over their subordinate siblings and how these factors can explain the evolution of siblicide in changing and unpredictable environments. Specifically, I investigate:

- which factors modulate variation in the control exerted by a dominant on its subordinate sibling’s access to maternal teats,
- whether behavioural training effects are implied in the emergence of a dominance relationship,
- whether females have a competitive advantage against males during sibling conflicts.

Chapter 3: Validates a new enzyme immunoassay to measure faecal glucocorticoid metabolites in spotted hyenas. The specific aims were to:

- test whether the assay used measured significant increases in fGCM after ACTH challenge and anaesthesia in captive animals,
- conduct a radio-metabolism study to characterise the fGCM detected by the assay,
- assess whether a predicted decrease in fGCM with increasing age was detected by the assay in faecal samples of free-ranging juvenile spotted hyenas in three large clans in the Serengeti National Park.

Chapter 4: Tests whether sibling rivalry and within-litter dominance status in juvenile spotted hyenas in the Serengeti National Park influence the level of fGCM in dominant and subordinate siblings, using the assay validated in Chapter 3.

Finally, in **Chapter 5** I summarise the key findings of this thesis, and discuss them in the broader context of sibling rivalry in bird and mammalian species.

1.8 References

Abbott DH, Keverne EB, Bercovitch FB, Shively CA, Mendoza SP, Saltzman W, Snowdon CT, Ziegler TE, Banjevic M, Garland T Jr, Sapolsky RM (2003) Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Hormones and Behaviour 43: 67–82.

Anderson DJ, Reeve J, Martinez Gomez JE, Weathers WW, Hutson S, Cunningham HV, Bird DM (1993) Sexual size dimorphism and food requirements of nestling birds. Canadian Journal of Zoology 71: 2541-2545.

Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby ML, Kennedy MW (2002) Carotenoids and egg quality in the lesser blackbacked gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proceedings of the Royal Society London B 269: 29–36.

Bortolotti GR (1986) Influence of sibling competition on nestling sex ratios of sexually dimorphic birds. American Naturalist 127: 495–507.

Chase ID, Bartolomeo C, Dugatkin LA (1994) Aggression interactions and inter-contest interval: how long do winners keep winning? Animal Behaviour 48: 393–400.

Chase ID, Tovey C, Spangler-Martin D, Manfredonia M (2002) Individual differences versus social dynamics in the formation of animal dominance hierarchies. Proceedings of the National Academy of Sciences of the USA 99: 5744-5749.

Cook MI, Monaghan P, Burns MD (2000) Effects of short-term hunger and competitive asymmetry on facultative aggression in nestling black guillemots *Cepphus grylle*. Behavioural Ecology 11: 282–287.

Dloniak SM, French JA, Holekamp, KE (2006) Rank-related maternal effects of androgens on behaviour in wild spotted hyaenas. Nature 440: 1190-1193.

van Doorn GS, Hengeveld GM, Weissing FJ (2003) The evolution of social dominance I: Two-player models. Behaviour 140: 1305-1332.

Drea CM, Hawk JE, Glickman SE (1996) Aggression decreases as play emerges in infant spotted hyaenas: preparation for joining the clan. Animal Behaviour 51: 1323–1336.

Drummond H, Gonzalez E, Osorno JL (1986) Parent-offspring cooperation in the blue-footed booby (*Sula nebouxii*): Social roles in infanticidal brood reduction. Behavioural Ecology and Sociobiology 19: 365-372.

Drummond H, Garcia Chavelas C (1989) Food shortage influences sibling aggression in the blue-footed booby. Animal Behaviour 37: 806–819.

Drummond H, Osorno JL, Torres R, García-Chavelas C, Merchant H (1991) Sexual size dimorphism and sibling competition: implication for avian sex ratios. American Naturalist 138: 623–664.

Drummond H, Osorno J L (1992) Training siblings to be submissive losers: dominance between booby nestlings. Animal Behaviour 44: 881–893.

Drummond H, Canales C (1998) Dominance between booby nestlings involves winner and loser effects. Animal Behaviour 55: 1669–1676.

Drummond H (2001) The control and function of agonism in avian broodmates. Advances in the Study of Behavior 30: 261-301.

Drummond H, Rodríguez C, Vallarino A, Valderrábano C, Rogel G, Tobón E (2003) Desperado siblings: uncontrollably aggressive junior chicks. Behavioral Ecology and Sociobiology 53: 287–296.

Drummond H (2006) Dominance in vertebrate broods and litters. The Quarterly Review of Biology 81: 3-32.

Drummond H, Rodríguez C, Schwabl H (2008) Do mothers regulate facultative and obligate siblicide by differentially provisioning eggs with hormones? Journal of Avian Biology 39: 139-143.

East ML, Hofer H (2002) Conflict and cooperation in a female-dominated society: a reassessment of the "hyperaggressive" image of spotted hyenas. Advances in the Study of Behavior 31: 1-30.

East ML, Höner OP, Wachter B, Wilhelm K, Burke T, Hofer H (2009) Maternal effects on offspring social status in spotted hyenas. Behavioral Ecology 20: 478-483.

Eising CM, Eikenaar C, Schwabl H, Groothuis TGG (2001) Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. Proceedings of the Royal Society London B 268: 839-846.

Enquist M, Leimar O (1987) Evolution of fighting behaviour: the effect of variation in resource value. Journal of Theoretical Biology 127: 187-205.

Fawcett TW, Johnstone RA (2010) Learning your own strength: winner and loser effects should change with age and experience Proceedings of the Royal Society London B 277: 1427-1434.

Ferin M (1999) Stress and the reproductive cycle. The Journal of Clinical Endocrinology and Metabolism 84: 1768–1774.

Fey K, Trillmich F (2008) Sibling competition in guinea pigs (*Cavia aperea f. porcellus*): scrambling for mother's teats is stressful. Behavioural Ecology and Sociobiology 62: 321–329.

Forbes LS, Ydenberg RC (1992) Sibling Rivalry in a Variable Environment. Theoretical Population Biology 41: 135-160.

Frank LG (1986) Social organization of the spotted hyaena (*Crocuta crocuta*). I. Demography. Animal Behaviour 34: 1500–1509.

Frank LG, Glickman SE, Licht P (1991) Fatal sibling aggression, precocial development, and androgens in neonatal spotted hyaenas. Science 252: 702–704.

Fraser D, Thompson BK (1991) Armed sibling rivalry among suckling piglets. Behavioural Ecology and Sociobiology 29: 9-15.

Fujioka M (1985) Sibling competition and siblicide in asynchronously hatching broods of the cattle egret *Bubulcus ibis*. Animal Behaviour 33: 1228-1242.

Godfray HCJ, Harper AB (1990) The evolution of brood reduction by siblicide in birds. Journal of Theoretical Biology 145: 163–175.

Golla W, Hofer H, East ML (1999) Within-litter sibling aggression in spotted hyaenas: effect of maternal nursing, sex and age. Animal Behaviour 58: 715-726.

Goymann W, Möstl E, Van't Hof T, East ML, Hofer H (1999) Non-invasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. General and comparative Endocrinology 114: 340-348.

Goymann W, East ML, Wachter B, Höner O, Möstl E, Van't Hof TJ, Hofer H (2001a) Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyaenas. Proceedings of the Royal Society London B 268: 2453-2459.

Goymann W, East ML, Hofer H (2001b) Androgens and the role of female “hyperaggressiveness” in spotted hyenas (*Crocuta crocuta*). Hormones and Behavior 39: 83-92.

Goymann W, Wingfield JC (2004) Allostatic load, social status and stress hormones: the costs of social status matter. Animal Behaviour 67: 591-602.

Grafen A (1987) The logic of divisively asymmetric contests: respect for ownership and the desperado effect. Animal Behaviour 35: 462–467.

Groothuis TGG, Müller W, von Engelhardt N, Carere C, Eising C (2005) Maternal hormones as a tool to adjust offspring phenotype in avian species. Neuroscience and Biobehavioral Reviews 29: 329-352.

Guerra M, Drummond H (1995) Reversed sexual size dimorphism and parental care: minimal division of labour in the blue-footed booby. Behaviour 132: 479-496.

Hamilton WD (1964) The genetical evolution of social behaviour. Journal of Theoretical Biology 7: 1-16.

Hammerstein P (1981) The role of asymmetries in animal contests. Animal Behaviour 29: 193-205.

Henry JD (1985) The little foxes. Natural History 94: 46-57

Hofer H, East ML (1993a) The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. I. Social organization. Animal Behaviour 46: 547–557.

Hofer H, East ML (1993b) The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. II. Intrusion pressure and commuters’ space use. Animal Behaviour 46: 559–574.

Hofer H, East ML (1993c) The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. III. Attendance and maternal care. Animal Behaviour 46: 575–589.

Hofer H, East ML (1995) Population dynamics, population size and the commuting system of Serengeti spotted hyenas. In: A.R.E. Sinclair, P. Arcese (eds) Serengeti II. Dynamics, management, and conservation of an ecosystem, pp 332-363. Chicago, University of Chicago Press.

Hofer H, East ML (1997) Skewed offspring sex ratios and sex composition of twin litters in Serengeti spotted hyaenas (*Crocuta crocuta*) are a consequence of siblicide. Applied Animal Behaviour Science 51: 307–316.

Hofer H, East ML (2003) Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective. Evolutionary Ecology 17: 315–331.

Hofer H, East ML (2008) Siblicide in Serengeti spotted hyenas: a long-term study of maternal input and cub survival. Behavioural Ecology and Sociobiology 62: 341-351.

Hofer H, East ML (2012) Stress and immunosuppression as factors in the decline and extinction of wildlife populations: the concepts, the evidence and the challenges. In: Aguirre AA, Daszak P, Ostfeld RS (eds) Conservation medicine: applied cases of ecological health, pp 82-107. Oxford University Press, New York.

Höner OP, Wachter B, Hofer H, Wilhelm K, Thierer D, Burke T, Trillmich F, East ML (2010) The fitness of dispersing spotted hyaena sons is influenced by maternal social status. Nature Communications 1: 60.

Hsu Y, Early L Wolf, LL (2006) Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. Biological Reviews 81: 33-74.

Hudson R, Trillmich F (2008) Sibling competition and cooperation in mammals: challenges, developments and prospects Behavioural Ecology and Sociobiology 62: 299–307.

James WH, Hofer H (1999) A note on sex ratios and sex combinations in Serengeti spotted hyaenas: siblicide and sub-binomial variance. Applied Animal Behaviour Science 65: 153-158.

Inoue Y (1985) The process of asynchronous hatching and sibling competition in the little egret *Egrettag arzetta.* Colonial Waterbirds 8: 1-12.

Khan MZ, Altmann J, Isani SS, Yu J (2002) A matter of time: evaluating the storage of fecal samples for steroid analysis. General and Comparative Endocrinology 128: 57–64.

Lack DL (1954) The natural regulation of animal numbers. Oxford, Oxford University Press.

Leippert D, Goymann W, Hofer H (2000) Between-litter siblicide in captive Indian false vampire bats, *Megaderma lyra*. Journal of Zoology London 251: 537-540.

Licht P, Frank LG, Pavgi S, Yalcinkaya TM, Siiteri PK, Glickman SE (1992) Hormonal correlates of 'masculinization' in female spotted hyaenas (*Crocuta crocuta*). 2. Maternal and fetal steroids. Journal of Reproduction and Fertility 95: 463-474.

Liprap RM (1993) Stress and reproduction in domestic animals. Annals of the New York Academy of Sciences 697: 275–284.

Machmer MM, Ydenberg RC (1998) The relative roles of hunger and size asymmetry in sibling aggression between nestling ospreys, *Pandion haliaetus*. Canadian Journal of Zoology 76: 181-186.

Macpherson AH (1969) The dynamics of Canadian arctic fox populations. Canadian Wildlife Service Report Series 8: 1-49.

Magrath MJL, Brouwer L, Komdeur J (2003) Egg size and laying order in relation to offspring sex in the extreme sexually size dimorphic brown songlark, *Cinclorhamphus cruralis*. Behavioural Ecology and Sociobiology 54: 240–248.

Mateo JM (2008) Inverted-U shape relationship between cortisol and learning in ground squirrels, Neurobiology of Learning and Memory 89: 582–590.

Mills, M.G.L. (1990) Kalahari hyaenas. The comparative behavioural ecology of two species. London: Unwin Hyman.

Mock DW (1985) Siblicidal brood reduction: the prey size hypothesis. The American Naturalist 125: 327-343.

Mock DW, Parker GA (1986) Advantages and disadvantages of egret and heron brood reduction. Evolution 40: 459-470.

Mock DW, Lamey TC, Ploger BJ (1987) Proximate and ultimate roles of food amount in regulating egret sibling aggression. Evolution 68: 1760-1772.

Mock DW, Drummond H, Stinson CH (1990) Avian siblicide. American Scientist 78: 438-449.

Mock DW, Parker GA (1997) The evolution of sibling rivalry. Oxford, Oxford University Press.

Monfort SL, Brown JL, Wildt DE (1993) Episodic and seasonal rhythms of cortisol secretion in male Eld’s deer (*Cervus eldi thamin*). Journal of Endocrinology 138: 41–49.

Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions, Endocrine Reviews 5: 25–44.

Nuñez de la Mora A, Drummond H, Wingfield J (1996) Hormonal correlates of dominance and starvation-induced aggression in chicks of the blue-footed booby. Ethology 102: 748-761.

Oddie KR (2009) Size matters: competition between male and female great tit offspring. Journal of Animal Ecology 69: 903-912.

Osorno JL, Drummond H (1995) The function of hatching asynchrony in the blue-footed booby. Behavioural Ecology and Sociobiology 35: 265-273

Palme R, Fischer P, Schildorfer H, Ismail MN (1996) Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Animal Reproduction Science 43: 43-63.

Parker GA (1974) Assessment strategy and the evolution of fighting behaviour. Journal of Theoretical Biology 47: 223–243.

Parker GA, Mock DW, Lamey TC (1989) How selfish should stronger sibs be? The American Naturalist 133: 846–868.

Pinson D, Drummond H (1993) Brown pelican siblicide and the prey-size hypothesis. Behavioural Ecology and Sociobiology 32: 111-118.

Ploger BJ, Mock DW (1986) Role of sibling aggression in the distribution of food to nestling cattle egrets (*Bubulcus ibis*). Auk 103: 768-776.

Pournelle GH (1965) Observations on the birth and early development of the spotted hyaena. Journal of Mammalogy 46: 503.

Pride RE (2005) High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). Biology Letters 1: 60-63.

Ramos-Fernandez G, Nunez-de la Mora A, Wingfield JC, Drummond H (2000) Endocrine correlates of dominance in chicks of the blue-footed booby (*Sula nebouxii*): Testing the challenge hypothesis. Ethology Ecology and Evolution 12: 27–34.

Rodríguez-Gironés MA, Drummond H, Kacelnik A (1996) Effect of food deprivation on dominance status in blue-footed booby (*Sula nebouxii*) broods. Behavioural Ecology 7: 82-88.

Romero ML (2004) Physiological stress in ecology: lessons from biomedical research. Trends in Ecology and Evolution 19: 249-255.

Romero ML, Dickens MJ, Cyr NE (2009) The reactive scope model—a new model integrating homeostasis, allostasis and stress. Hormones and Behaviour 55: 375–389.

Sapolsky RM (1982) The endocrine stress-response and social status in the wild baboon. Hormones and Behavior 16: 279–292.

Sapolsky RM (2005) The influence of social hierarchy on primate health. Science 308: 648-652.

Sasvari L, Hegyi Z, Peczely P (1999) Brood reduction in white storks mediated through asymmetries in plasma testosterone concentrations in chicks. Ethology 105: 569–582.

Schwabl H (1993) Yolk is a source of maternal testosterone for developing birds. Proceedings of the National Academy of Sciences of the USA 90: 11446-11450.

Schwabl H, Mock DW, Gieg JA (1997) A hormonal mechanism for parental favouritism. Nature 386: 231.

Smale L, Frank LG, Holekamp KE (1993) Ontogeny of dominance in free-living spotted hyaenas: juvenile rank relations with adult females and immigrant males. Animal Behaviour 46: 467–477.

Smale L, Holekamp KE, Weldele M, Frank LG, Glickman SE (1995) Competition and cooperation between littermates in the spotted hyaena *(Crocuta crocuta*). Animal Behaviour 50: 671-682.

Smale L, Holekamp KE, White PA (1999) Siblicide revisited in the spotted hyaena: does it conform to obligate or facultative models? Animal Behaviour 58: 545-551.

Sockman KW, Schwabl H (2000) Yolk androgens reduce offspring survival. Proceedings of the Royal Society London B 267: 1451–1456.

Spear L, Nur N (1994) Brood size, hatching order and hatching date: Effects on 4 life-history stages from hatching to recruitment in western gulls. Journal of Animal Ecology 63: 283-298.

Stockley P, Parker GA (2002) Life history consequences for mammalian sibling rivalry. Proceedings of the National Academy of Sciences of the USA 99: 12932–12937.

Tarlow EM, Wikelski M, Anderson D (2001) Hormonal correlates of siblicide in Galápagos Nazca boobies. Hormones and Behaviour 40: 14-20.

Teather KL (1992) An experimental study of competition for food between male and female nestlings of the red-winged blackbird. Behavioural Ecology and Sociobiology 31: 81-87.

Touma C, Palme R (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Annals of the New York Academy of Sciences 1046: 54-74.

Trillmich F, Wolf JBW (2008) Parent–offspring and sibling conflict in Galápagos fur seals and sea lions. Behavioural Ecology and Sociobiology 62: 363-375.

Verboven N, Monaghan P, Evans DM, Schwabl H, Evans N, Whitelaw C, Nager RG (2003) Maternal condition, yolk androgens and offspring performance: a supplemental feeding experiment in the lesser blackbacked gull (*Larus fuscus*). Proceedings of the Royal Society London B 270: 2223–2232.

Wachter B, Höner OP, East ML, Golla W, Hofer, H (2002) Low aggression levels and unbiased sex ratios in a prey-rich environment: no evidence of siblicide in Ngorongoro spotted hyenas (*Crocuta crocuta*). Behavioural Ecology and Sociobiology 52: 348–356.

Wada H (2008) Glucocorticoids: Mediators of vertebrate ontogenetic transitions. General and Comparative Endocrinology 156: 441-453.

Wahaj SA, Holekamp KE (2006) Functions of sibling aggression in the spotted hyaena, *Crocuta crocuta*. Animal Behaviour 71: 1401-1409.

Wasser SK, Bevis K, King G, Hanson E (1997) Noninvasive physiological measures of disturbance in the northern spotted owl. Conservation Biology 11: 1019–1022.

Werschkul DF, Jackson JA (1979) Sibling competition and avian growth rates. Ibis 121: 97-102.

Williams TD (1994) Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. Biological Reviews 68: 35–59.

Wingfield JC, Deviche P, Sharbaugh S, Astheimer LB, Holberton R, Suydam R, Hunt K (1994) Seasonal changes of the adrenocortical responses to stress in redpolls, *Acanthis flammea*, in Alaska. Journal of Experimental Zoology 270: 372–380.

CHAPTER 2

Sibling rivalry: training effects, emergence of dominance and incomplete control

(in revision)

SARAH BENHAIEM, HERIBERT HOFER, STEPHANIE KRAMER-SCHADT,

EDGAR BRUNNER, MARION L. EAST

Author contribution:

Conceptual framework and predictions of this study were discussed and developed by Sarah Benhaiem (SB), Marion L. East (ME) and Heribert Hofer (HH). A majority of the video focal samples were recorded by SB and some by ME. Focal samples were analysed on the software INTERACT by SB. The framework of the model (GLMM) on R was programmed by Stephanie Kramer-Schadt (SKS), applied and adapted by SB. SB did the other statistical analyses using R except for those using SYSTAT (survival analysis with censored data) which were done by HH, and except the non-parametric model which was developed by Edgar Brunner (EB) on SAS. The manuscript was written by SB, extensively commented on and edited by ME and also edited by SKS and HH.

Sibling rivalry: training effects, emergence of dominance and incomplete control

SARAH BENHAIEM [1], HERIBERT HOFER [1], STEPHANIE KRAMER-SCHADT [1], EDGAR BRUNNER [2], MARION L. EAST [1]

[1] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

[2] University of Göttingen, Faculty of Medicine, Department of Medical Statistics, Humboldtallee 32, D-37073 Göttingen, Germany

Corresponding author: benhaiem@izw-berlin.de,

phone: +49-30-51-68-520

http://rspb.royalsocietypublishing.org/content/early/2012/06/15/rspb.2012.0925

Abstract: Within-brood or litter dominance provides fitness-related benefits if dominant siblings selfishly skew access to food provided by parents in their favour. Models of facultative siblicide assume that dominants exert complete control over their subordinate sibling's access to food and that this control is maintained irrespective of the subordinate's hunger level. In contrast, the desperado sibling hypothesis suggests subordinates contest access to food when the cost of not doing so is high. Here we show that within spotted hyena (*Crocuta crocuta*) twin litters, dominants most effectively skew access to maternal milk in their favour when their aggression prompts a highly submissive response. When hungry, subordinates were less submissive in response to aggression, thereby decreasing lost suckling time and increasing suckling time lost by dominants. In a species where adult females socially dominate adult males, juvenile females were more often dominant than males in mixed-sex litters, and subordinate sisters used more effective counter-tactics against dominant brothers than subordinate brothers against dominant sisters. Our results provide the first evidence in a mammal that dominant offspring in twin litters do not exert complete control over their sibling's access to resources (milk), and that sibling dominance relationships are influenced by sibling sex and training effects.

Key words: sibling rivalry, spotted hyena, aggression, dominance, training effects

1. INTRODUCTION

Although siblings may provide each other with indirect fitness benefits [1], within-brood or litter asymmetric competition may result in fitness costs to the subordinate and fitness benefits for the dominant sibling [2]. Dominants that selfishly consume the largest share of the food provided by parents can benefit from increased growth and survival whereas subordinates may suffer reduced growth and survival [3,4]. Theoretically, siblings should avoid escalated conflicts by adhering to conventions based on differences in resource holding power, unless the benefits outweigh their costs [5,6]. Competitive asymmetries may be conferred by birth order in avian species with marked asynchronous hatching [7,8], or by intrinsic factors such as fighting ability [9,10,11]. Alternatively, dominance may result from arbitrary historical asymmetries such as behavioural training effects, in which the experience of winning or losing a contest alters an individual's probability of winning or losing future contests [12,13]. Few studies have investigated these effects in mammals (but see [14]) and we know of no study that demonstrated this phenomenon in a free-ranging mammal.

Avian models of facultative siblicide predict that as parental food provisioning rates decline, dominant chicks should aggressively increase their share of food [2,5,6]. Such a response occurs in several bird species [7,8,13] and at least one mammal, the spotted hyena *Crocuta crocuta* [4], and may lead to facultative siblicide through enforced starvation or physical damage [2]. The models assume that dominants have complete control over the share of food received by subordinates, regardless of the subordinates' hunger level. As death might result from a hungry chick's submissiveness, selection should act against subordinates accepting complete control by dominants [15]. Extending the idea of the "desperado effect" [16], the desperado sibling hypothesis [17] describes extreme aggressiveness by subordinate chicks without survival prospects in obligatory siblicidal species. A recent hypothesis [18] suggests that in facultatively siblicidal species, subordinates should adjust their level of submission to their survival prospects and increase their assertiveness towards dominants as the cost of subordination in terms of likelihood of starvation increases.

We investigated sibling rivalry over access to maternal teats in a social carnivore, the spotted hyena. We focus on aggression by dominants to terminate their subordinate's access to teats and the responses of subordinates to this aggression. We quantify the degree of skew dominants achieve in terms of access to teats, test whether trained loser effects contributed to the emergence of the within-litter dominance relationship, and explore how hunger and sex affect the competitiveness of dominants and subordinates.

2. STUDY SPECIES AND PREDICTIONS

Spotted hyena females live in linear dominance hierarchies, socially dominate males and give birth to usually one or two cubs [4]. Cubs completely depend on nutritious maternal milk [19] during their first six months, with lactation lasting up to 18 months [19,20]. Female spotted hyenas have two functional teats, and as in other mammals [21], siblings of a twin litter are nursed simultaneously. There is intense rivalry between littermates [22,23], probably because dominants achieve higher growth and survival than subordinates [4,24], which may lead to facultative siblicide through enforced starvation [4, 25]. Aggression begins within hours after birth between cubs with open eyes and teeth erupted [26], results in the establishment of dominance and declines with age [22]. During suckling, dominants lie parallel to their mother's belly and subordinates typically between her hind legs (suppl. figure 1). To stop their sibling suckling, dominants have to terminate their own milk intake. Because littermates cannot simultaneously suckle and engage in aggressive interactions, both would benefit from a behavioural convention according to which subordinates submit to dominants. As a corollary, aggression should cause only a short break in suckling for dominants and a longer break for subordinates, depending on their precise behavioural response. As shown for dominance hierarchies in principle [27] and for chicks [13], trained loser effects during repeated aggressive encounters in which subordinates gradually learn to submit to dominants can establish such a behavioural convention without requiring body size asymmetries (see ESM 1) or other differences in resource holding power. Evidence for trained effects would include increasingly submissive responses from subordinates to repeated aggression by dominants [12] and be most apparent when littermates are young and dominance relationships less stable [22].

In the Serengeti ecosystem, Tanzania, fluctuating prey abundances provide an opportunity to assess the effect of declining provisioning rates on the aggressive behaviour of dominants and the assertiveness of subordinates in reply. Such fluctuations inside the group territory through the presence or absence of major migratory herds of ungulates [28,29] are responsible for a social status-linked central-place foraging system of hyenas. With major herds present, all mothers forage inside the group territory and nurse daily [19]. When moderately-sized herds are present, mothers of high social status and some of medium status continue to forage inside whereas lower ranking females commute to distant major herds for periods of one to several days [30]. When all herds are elsewhere, all mothers commute long distances and nurse their offspring only once every few days [4,19]. As maternal absence intervals increase, hungry dominants should aggressively seek to decrease access of the subordinate to maternal teats, whereas hungry subordinates should be less willing to adhere to conventions, be more assertive [18] and resume suckling faster after aggression. In response to such a challenge by subordinates, hungry dominants should escalate aggression [22,31], which should reduce their suckling time.

In our study population, the proportion of males at three months of age after the age at which siblicide usually occurred [25,32] showed no bias (0.52, [25]), but masked a female bias amongst singletons. This suggests that females in both same-sex [32] and mixed-sex litters survived intense sibling rivalry more often than males and may be better at monopolising teats than males. If so, then in mixed-sex litters there should be a female bias amongst dominants and subordinate females should be more successful at reversing dominance than males. If a dominant sister is a better competitor than a dominant brother, hungry and more "at risk" subordinates should be more assertive against dominant sisters than against dominant brothers.

3. METHODS

(**a**) ***Study population and behaviour***

The study was conducted as part of an ongoing long-term study between 2007 and 2009 on three clans in the centre of Serengeti National Park, Tanzania. Individuals were recognised by their spot patterns [28, 33], cubs without spots by ear notches, scars or bald patches [22]. Age and sex were determined using standard methods (ESM 1). Twin litters were observed at clan communal dens during periods of several hours around dawn and dusk. We recorded 97 video focal samples of 15 minutes duration [34] at the start of the first suckling session, when aggression is highest, after a mother arrived at a den [22]. We analysed sibling behaviours in 38 twin litters from 34 different mothers (mean focal samples per litter 2.6 ± 1.7, range 1-7). Litters were aged between 9 and 346 days; 50% of focal samples were from litters less than 81 days (ESM 1). We measured growth rates of paired dominants and subordinates in 13 twin litters aged approximately 90 days as described by [19]. We calculated the average duration of suckling bouts after a mother returned from 115 suckling bouts of 45 females in 84 twin litters.

(**b**) ***Predictor variables***

(i) *Hunger level*

We used three predictor variables known to significantly affect frequency of nursing and cub growth rates [19,30]: prey abundance, maternal den attendance and maternal social status (standardised rank). Standardised rank (*mstatus*, ESM 1) was calculated [35] by evenly distributing adult female ranks between the highest (+1) and lowest rank (-1), with the median rank being 0. Prey abundance (*prey*) was scored [28] for each observation period as low (~3.3 animals km^{-2}), medium (~31 animals km^{-2}) or high (~238 animals km^{-2} (details in ESM 1)). Maternal den attendance was extracted from detailed records of all individuals within a 100 m radius of communal dens during dawn and dusk observation periods [4,19,30, ESM 1]. Cubs were defined as "fed" when mothers were observed at the den during a 30h period prior to the focal sample and in two focal samples during high prey abundance when cub bellies indicated they had been recently fed. Cubs were defined as "hungry" when mothers were not seen at the den in the previous 30h during medium and low prey abundance and known to be absent on long-distance foraging trips [29, details in ESM1].

(ii) *Sibling tactics*

We scored aggressive behaviour by dominants (*domscore*) against their suckling subordinate sib as (+1) low, (+2) moderate and (+3) high intensity aggression. We scored the submissiveness of the subordinate's response (*subscore*) during the following 5 seconds as (-2) highly submissive, (-1) marginally submissive, (0) no response, (+1) marginally aggressive and (+2) moderately aggressive (details see ESM 1, suppl. table 2). Dominants initiated 94.5% (1053 of 1115) of agonistic interactions. We calculated for each focal sample mean scores for the dominant's behaviours (*mean.domscore*) and the subordinate's response (*mean.subscore*). A high *mean.domscore* indicated high intensity aggression, a high *mean.subscore* assertive or aggressive responses and a low *mean.subscore* highly submissive responses.

(c) ***Dependent variables***

(i) *Time lost suckling*

We measured the time (latency) elapsed after each aggressive act by the dominant until each sibling resumed suckling and calculated means per focal sample for the dominant and subordinate cub (mean latencies *domlat* and *sublat*). If a subordinate did not resume suckling within 15 minutes after aggression the subordinate's latency was scored as a minimum of 900 seconds (N = 5 focal samples in 5 litters).

(ii) *Relative delay*

For each focal sample we calculated the relative delay (*rel.delay*) between siblings as the standardised proportion of time lost suckling by the subordinate (*sublat*) relative to that of the dominant: *rel. delay* = 1- (*domlat* / *sublat*). When *rel. delay* tended towards + 1, dominants had a strong competitive advantage because subordinates took far longer to resume suckling than the dominants (*sublat* >>*domlat*). In 7 of 97 focal samples *domlat* was slightly higher than *sublat*. In these cases we set *rel. delay* to 0, i.e., no relative delay for the subordinate.

(iii) *Loser effect*

We used focal samples with a minimum of 17 interactions (20 focal samples, 14 litters, details in ESM 1) to test for a trained loser effect. A loser effect would require a significant increase in the submissiveness of the subordinate's response (*subscore*) during a focal sample, therefore produce a decrease in the value of *subscore* and a negative slope in a linear regression of *subscore* against time. We also tested for an increase of regression slopes with age (after logarithmic transformation (to base 10) of age to obtain a linear relationship) to see whether loser effects were more apparent in younger than older litters. The residuals of these regressions did not deviate from normal distribution (Lilliefors test) and variances showed no evidence of heterogeneity (residual plots).

(iv) *Dominance and its reversal in twin litters*

Data from mixed-sex litters from 1989 until 2011 in the study clans were used to test whether (1) females were more often dominant than males and (2) subordinate females gained dominance more often than subordinate males during dominance reversals.

(**d**) ***Statistical analysis***

Unless stated otherwise, we used R (R Development Core Team, v.2.11.1), set the threshold for significance at 5% and refer to numbers of litters as sample size. All tests were two-tailed. Statistics are quoted as means ± SD. We calculated mean latencies from non-parametric Kaplan – Meier survivorship functions [36] in Systat 13.0 (Systat Inc., San Jose, CA, USA) by incorporating right-censored data when subordinates did not resume suckling within at least 15 minutes. For each litter we calculated mean latencies for dominants (*domlat*) and subordinates (*sublat*) from the means per focal sample to avoid pseudoreplication. We applied Wilcoxon signed-rank tests to compare mean latencies of dominants and subordinates and growth rates of dominants and their paired subordinate siblings at 90 days of age. In mixed-sex litters, we tested deviations from unity in the sex ratio of dominants and the sex ratio of subordinates successful in reversing dominance relationships by applying an exact binomial test, the latter comparison based on all litters in which dominance reversals occurred.

We used generalised linear mixed models (lme4 library of R ([37]) to assess the influence of our predictors on the dependent variables (1) *rel. delay*, (2) *domlat* and (3) *sublat* (details in ESM 1), with litter identity as random factor to avoid overrepresentation of individual litters. We used an information-theoretic approach to compare fitted models and select those with the smallest values for the Akaike Information Criterion (AIC). When two models differ in their AIC values by less than 2, both can be considered competitive for interpretation [38]. In this case, we present and interpret (with caution) the effects of the variables included in the most complete model (when two nested models were compared) as they had a strong biological relevance (details in ESM 1). The residuals of all presented models did not deviate from normal distribution (Lilliefors test) and variances were not heterogeneous (residual plots). Supplementary table 3 (ESM 2) contains estimated coefficients and standard errors for the selected models.

We tested whether the assertiveness of the subordinate (*subscore*) in hungry and fed litters depended on the sex of the dominant by extending a non-parametric hierarchical mixed model [39,40] to a two-way layout for hunger level and sex of dominant sibling, with focal samples nested within litters as random factor, and implemented it in SAS v9.3. For a majority of litters, focal samples were available only at one hunger level. If focal samples of a litter were recorded at both hunger levels then we only included the hunger level where the highest number of agonistic interactions occurred to ensure independence of data points. Values of *subscore* were ranked to obtain mean ranks for each litter. A post-hoc analysis stratified on hunger level was used to test the effect of the sex of the dominant at each category of hunger level. Significance threshold was adjusted to 0.025.

4. RESULTS

(a) *Lost suckling time*

When dominants stopped suckling to aggressively exclude subordinates from teats, their mean latency to resume suckling was shorter than that of subordinates (all ages; dominants: 3.0 ± 2.5 s, subordinates: 48.2 ± 114.1 s, Wilcoxon signed-rank test: $V = 30$, $N = 38$, $P < 0.0001$). This difference was extreme in younger (<120 days of age; dominants: 3.3 ± 2.7 s, subordinates: 66.4 ± 138.2 s, $V = 14$, $N = 24$, $P < 0.0001$) and still apparent in older litters (>120 days of age; dominants: 2.6 ± 2.2 s, subordinates: 17.0 ± 40.4 s, $V = 3$, $N = 14$, $P = 0.003$). Without the five focal samples in which the subordinates did not resume suckling, the differences remained (all ages; dominants: 3.2 ± 2.8 s, subordinates: 10.0 ± 10.1 s, $V =34$, $N = 38$, $P < 0.0001$) and were still apparent within both age classes (litters < 120 days; dominants: 3.6

± 3.0 s, subordinates: 12.2 ± 10.7 s, V = 17, N = 24, $P < 0.0001$, suppl. figure 2a; litters > 120 days; dominants : 2.6 ± 2.2 s, subordinates: 6.3 ± 7.9 s, V = 3, N = 14, $P = 0.003$, suppl. figure 2b).

(**b**) ***Sibling growth rates and suckling duration***

Mean growth rates of dominants (88.5 ± 26.3 g/day) were significantly higher (V = 84, N = 13 litters, $P = 0.005$) than that of their paired subordinate siblings (81.4 ± 23.8 g/day) at a mean age of 83.4 ± 3.5 days for dominants (median 84.3) and 81.8 ± 5.6 days for subordinates (median 84.7). Average duration of suckling periods was 56 ± 38 min (N= 45 females).

(**c**) ***Relative delay (skew by dominants)***

The mean relative delay between siblings was 0.5 ± 0.3, i.e., dominants returned to suckle 50% faster than subordinates. When the skew was low (between 0 and 0.2; 24 focal samples), mean suckling time lost by dominants was high (4.9 ± 3.9 s). When the skew was high (equal or higher than 0.8; 24 focal samples), mean suckling time lost by dominants was short (1.6 ± 1.2 s). Relative delay declined as (1) subordinates increasingly used assertive behaviours in response to aggression by dominants (figure 1a), (2) when litters were hungry (figure 1b) and (3) as age increased within the first 120 days after birth (figure 1c, “model 1”, table 1, ESM 2).

(**d**) ***Suckling time lost by dominants***

Suckling time lost by dominants owing to their own aggression increased as the (1) mean suckling time lost by subordinates increased (figure 2a), (2) intensity of aggression of dominants increased (figure 2b), (3) age increased (figure 2c), and (4) assertiveness of subordinate responses increased, especially when the subordinate was female and the dominant was male (figure 2d, top right). Dominants lost suckling time more slowly with increased assertiveness of subordinate responses in all-female litters (figure 2d, bottom left) and when the subordinate was male and the dominant was female (figure 2d, top left, “model 2”, table 1, ESM 2).

(**e**) ***Suckling time lost by subordinates***

Suckling time lost by subordinates following aggression by dominants (1) increased as the mean suckling time lost by dominants increased (figure 3a), (2) decreased with increasing assertiveness of subordinates (figure 3b), (3) decreased with age (figure 3c), declined with increasing aggressiveness of dominants when (4) the subordinate was male and the dominant was female (figure 3d) and (5) in fed litters (figure 3e, “model 3”, table 1, ESM 2).

(f) *Subordinate assertiveness*

As the sex of the dominant significantly changed how hunger level affected the assertiveness of the subordinate ($F_{1,18.5} = 9.08$, N = 38, $P = 0.007$), we performed a post-hoc analysis for the effect of the sex of the dominant stratified at each category of hunger level. Within hungry litters, subordinates with a dominant sister were significantly more assertive than subordinates with a dominant brother ($F_{1,17} = 6.81$, N = 19, $P = 0.018$), whereas within fed litters, assertiveness of subordinates was independent of the sex of dominants ($F_{1,17} = 2.80$, N = 19, $P = 0.113$, suppl. figure 3).

(g) *Loser effect*

All slopes of the regression of the intensity of submissive response against time per focal sample were highly significant. Intensity strongly increased during focal samples in response to repeated aggression in younger litters, resulting in strongly negative regression slopes (figure 4) and indicating a substantial loser effect. With increasing age, the regression slopes of *subscore* against time increased, indicating that trained loser effects were reduced or did not occur in older litters (log transformed linear regression against age, $F_{1,18} = 12.93$, $P = 0.002$, adjusted $r^2 = 0.39$).

(h) *Dominance and reversals in mixed-sex litters*

In 66.5% (121/182) of mixed-sex twin litters, the dominant was female, a significant bias (exact binomial test: $P = 0.0001$, 95% confidence interval (CI95%): 59.1 to 73.3%). Dominance reversals occurred in 7.0 % (27/388) of twin litters. In mixed-sex litters, 61.5% (8/13) of subordinates gaining dominance were female, and although this proportion did not deviate from chance, it was in the expected direction (exact binomial test: $P = 0.58$, CI95%: 32.7 to 83.4%).

5. DISCUSSION

Our results demonstrate that in spotted hyena twin litters dominants skew access to maternal teats by using periodic aggression against subordinates during suckling periods. Dominants achieved the highest skew when subordinates were highly submissive (figure 1, 2), when litters had been recently nursed (figures 1, 3) and when young (figure 1). Interestingly, in this female-dominated species, counter-tactics by assertive subordinate sisters were more effective against dominant brothers than counter-tactics by subordinate brothers against dominant sisters (figure 2d). Dominant sisters were highly challenged by subordinates when hungry, suggesting that subordinates with dominant sisters face a greater risk of siblicide than siblings with dominant brothers (suppl. figure 3, [25,32]). The behaviours between competing littermates were modified by sibling sex, age and hunger, included trained loser effects, behavioural conventions and assertive counter-tactics by hungry subordinates.

When parents feed their offspring with frequent solid food items, as in several birds or mammals (e.g., [41]), sibling competition is not fatal and may be ameliorated through begging. By contrast, in spotted hyenas, Galápagos fur seals (*Arctocephalus galapagoensis*) and sea lions (*Zalophus wollebaeki*) [42], offspring dependence on maternal milk is exceptionally long, and lactating females leave their offspring for several days during foraging trips. In such systems, low and fluctuating levels of resources result in infrequent and unpredictable nursing periods, favouring the evolution of intense sibling rivalry and facultative siblicide [2]. Subordinate spotted hyena siblings were excluded from maternal teats for substantially more time than dominants. As dominants at three months (this study) and at six months [4,24] grew significantly faster, aggressive exclusion of subordinates is likely to reduce their milk intake below that of dominants. Hofer et al. (unpublished data) showed that in matched measurements, milk intake rate during a suckling bout was significantly higher for dominants (9.0 ± 2.2 g min^{-1}) than subordinates (5.8 ± 1.4 g min^{-1}). The average delay of a subordinate to return to suckling after aggression by the dominant of 6.8 s therefore implies a loss of 657 mg of milk or the equivalent of 6.37 kJ on each occasion. Hence, even a small skew in lost suckling time ultimately provides a significant increase in growth rate for the dominant.

Provided subordinates were recently fed, they adhered to the behavioural convention and responded submissively when challenged by dominants, were easily excluded from teats (figure 3e, left) and dominants achieved a higher skew in terms of access to teats (figure 1b). Four (of 5) litters in which the subordinate did not resume suckling for at least 15 minutes after aggression had been recently nursed. This is in accordance with the hypothesis [18] that subordinates should abide by an established dominance convention provided the cost of doing so is not too large.

To subordinates, the cost of respecting convention should rise with hunger [18]. If subordinates strictly adhered to conventions even when hungry, despotic dominants (which may also be hungry) might consume most or all milk provided. If maternal input is low and mothers absent for several days, subordinates starve [4]. Hungry subordinates responded less submissively when challenged by dominants, thereby decreasing lost suckling time and increasing that of dominants. Such responses incited dominants to use lengthier and more intense aggression such as bite-shakes and chases. Hence, interactions between hungry sibs developed into escalated conflicts, resulting in an increase in lost suckling times for both (figures 2a, 3a). In 7.2% of focal samples, subordinates resumed suckling more quickly than dominants after aggression; in these cases aggression failed to skew suckling in the dominant's favour. The majority of these cases (5/7 focal samples; 4 litters) were observed in hungry litters with particularly thin cubs. The remaining two samples were recorded when prey abundance was low and mothers foraging on distant concentrations of migratory prey. In contrast to aggressive competition in some birds (e.g. [7, 8]), our results illustrate that aggression by dominants sometimes fails to provide clear benefits and are consistent with the idea that subordinate spotted hyena siblings facultatively adjust their agonistic behaviour to varying costs of subordination determined by ecological conditions [18].

Young subordinates significantly increased the intensity of their submission and delayed resumption of suckling when repeatedly attacked by dominants. This suggests a behavioural process akin to a trained loser effect in young litters and may explain why (1) young dominants achieved the highest skew in terms of access to teats, and (2) with increasing age, dominance relationships acquired stability because subordinates were submissive from the beginning of a suckling bout. It is also consistent with a recent model [43]which predicted training effects to be more pronounced in young and naive individuals if they were unaware of but willing to assess their own fighting ability. It is also likely that dominants benefitted from a trained winner effect because the subordinate became increasingly submissive. Young and hungry dominants may escalate aggression against subordinates during suckling bouts if the subordinate's behaviour is not sufficiently submissive – in other words, dominants "punish" ([31]) subordinates until their response and delay to resume suckling is sufficient. When older and fed, subordinates know they stand to lose less suckling time by responding appropriately to initial aggression by the dominant than prompting a series of escalating punishments by not doing so.

Owing to presumed sex asymmetries in competitiveness, it was repeatedly reported that during conflicts females are weaker competitors than males (e.g. [44]). This is clearly not the case in spotted hyena litters. Indeed, subordinate sisters were more effective than subordinate brothers in increasing suckling time lost by dominants. In 5 (of 6) litters in which dominants lost more suckling time than subordinates, the subordinates were females. Hence, subordinate females may be especially effective in countering aggression, particularly aggression by dominant brothers (figure 2d). In 66.5% of mixed-sex twin litters, females were dominant and subordinate females tended to reverse litter dominance order more frequently (61.5%) than subordinate males. As dominants grow faster than subordinates [4] and age at parturition of adult females declined with higher cub growth rate [30], it is possible that fitness benefits of dominance are higher for female than male cubs. Subordinates with dominant sisters increased their level of resistance during conflicts when their hunger level increased (suppl. figure 3), suggesting that females are better competitors than males.

During conflicts, dominants in spotted hyena litters trade-off sibling aggression and suckling. We studied sibling rivalry in a dynamic framework of interactions and addressed the roles of both dominants and subordinates in shaping the dominance relationship. Unlike the despotic control exerted by dominants on the reproduction of subordinates in species with a high reproductive skew (e.g., [45]), dominant spotted hyena siblings do not exert complete control over their littermate's access to maternal teats. Instead, there is substantial variation in the degree of control and this control is a dynamic rather than static state. In some litters, dominants reared during years when overall cub growth rates were low dispatched subordinates in 9% of litters through enforced starvation [4, 25]. In others, the long-term stability of dominance relationships was successfully undermined by (possibly assertive and hungry) subordinates and led to dominance reversals. The advantage of dominants during competition is therefore not absolute and depends on the interplay of age, maternal input, the sexes of both siblings and their behavioural tactics.

ACKNOWLEDGEMENTS

We are grateful to COSTECH for permission to conduct the study, and TAWIRI for their support of the research. We thank two referees and the associate editor for constructive and helpful comments, A. Francis, T. Shabani, D. Thierer, K. Wilhelm for assistance and the IZW for funding.

REFERENCES

1 Hamilton, W.D. 1964 The genetical evolution of social behaviour. *J. Theor. Biol.* **7**, 1-16. (doi:10.1016/0022-5193(64)90038-4)

2 Mock, D.W. & Parker, G. A. 1997 *The Evolution of Sibling Rivalry*. Oxford: Oxford University Press.

3 Fraser, D. & Thompson, B.K. 1991 Armed sibling rivalry among suckling piglets. *Behav. Ecol. Sociobiol.* **29**, 9-15. (doi:10.1007/BF00164289)

4 Hofer, H. & East, M.L. 2008 Siblicide in Serengeti spotted hyenas: a long-term study of maternal input and cub survival. *Behav. Ecol. Sociobiol.* **62**, 341-351. (doi:10.1007/s00265-007-0421-3)

5 Parker, G. A., Mock, D. W. & Lamey, T. C. 1989 How selfish should stronger sibs be? *Am. Nat.* **133,** 846–868. (doi:10.1086/284956)

6 Godfray, H.C.J. & Harper, A.B. 1990 The evolution of brood reduction by siblicide in birds. *J. Theor. Biol.* **145**, 163–175. (doi:10.1016/S0022-5193(05)80122-5)

7 Machmer, M.M & Ydenberg, R.C. 1998 The relative roles of hunger and size asymmetry in sibling aggression between nestling ospreys, *Pandion haliaetus. Can. J. Zool.* **76**, 181-186.

8 Cook, M.I., Monaghan P. & Burns, MD. 2000 Effects of short-term hunger and competitive asymmetry on facultative aggression in nestling black guillemots *Cepphus grylle*. *Behav. Ecol.* **11**, 282–287. (doi:10.1093/beheco/11.3.282)

9 Parker, G. A. 1974 Assessment strategy and the evolution of fighting behaviour. *J. Theor. Biol.* **47**, 223–243. (doi:10.1016/0022-5193(74)90111-8)

10 Hammerstein, P. 1981 The role of asymmetries in animal contests. *Anim. Behav.*, **29**, 193-205. (doi:10.1016/S0003-3472(81)80166-2)

11 Enquist, M. & Leimar, O. 1987 Evolution of fighting behaviour: the effect of variation in resource value. *J. Theor. Biol.* **127**, 187-205. (doi:10.1016/S0022-5193(87)80130-3)

12 Drummond, H. & Canales, C. 1998 Dominance between booby nestlings involves winner and loser effects. *Anim. Behav.* **55**, 1669-1676. (doi:10.1006/anbe.1997.0714)

13 Chase, I.D., Bartolomeo, C. & Dugatkin, L.A. 1994 Aggression interactions and inter-contest interval: how long do winners keep winning? *Anim. Behav.* **48**, 393–400. (doi:10.1007/s00265-010-1030-0)

14 Hsu, Y., Early, L. & Wolf, L.L. 2006 Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. *Biol. Rev.* **81**, 33-74. (doi: 10.1017/S146479310500686X)

15 Rodríguez-Gironés, M. A., Drummond, H. & Kacelnik, A. 1996 Effect of food deprivation on dominance status in blue-footed booby (*Sula nebouxii*) broods. *Behav. Ecol.* **7**, 82-88.

16 Grafen, A. 1987 The logic of divisively asymmetric contests: respect for ownership and the desperado effect. Anim. Behav. **35**, 462–467. (doi:10.1016/S0003-3472(87)80271-3)

17 Drummond, H., Rodríguez, C., Vallarino, A., Valderrábano, C., Rogel, G., Tobón, E. 2003 Desperado siblings: uncontrollably aggressive junior chicks. *Behav. Ecol. Sociobiol.* **53**, 287-296.

18 Drummond, H. 2006 Dominance in vertebrate broods and litters. *Q. Rev. Biol.* **81**, 3-32.

19 Hofer, H. & East, M.L.1993c The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. III. Attendance and maternal care. *Anim. Behav.* **46**, 575–589. (doi:10.1006/anbe.1993.1224)

20 Hofer, H. & East, M.L. 1995 Population dynamics, population size and the commuting system of Serengeti spotted hyenas. In *Serengeti II. Dynamics, Management, and Conservation of an Ecosystem (ed. A.R.E. Sinclair, P. Arcese)*, pp. 332-363. Chicago: University of Chicago Press.

21 Hudson, R. & Trillmich, F. 2008 Sibling competition and cooperation in mammals: challenges, developments and prospects. *Behav. Ecol. Sociobiol.* **62,** 299-307.

22 Golla, W., Hofer, H. & East, M.L. 1999 Within-litter sibling aggression in spotted hyaenas: effect of maternal nursing, sex and age. *Anim. Behav.* **58**, 715-726. (doi:10.1006/anbe.1999.1189)

23 Smale, L., Holekamp, K.E. & White, P.A. 1999. Siblicide revisited in the spotted hyaena: does it conform to obligate or facultative models? *Anim. Behav.* **58**, 545-551. (doi:10.1006/anbe.1999.1207)

24 Höner, O.P., Wachter, B., Hofer, H., Wilhelm, K., Thierer, D., Burke, T., Trillmich, F. & East, M.L. 2010 The fitness of dispersing spotted hyaena sons is influenced by maternal social status. *Nat. Commun.*, **1**, 60. (doi:http://dx.doi.org/10.1038/ncomms1059)

25 Hofer, H. & East M.L. 1997 Skewed offspring sex ratios and sex composition of twin litters in Serengeti spotted hyaenas (*Crocuta crocuta*) are a consequence of siblicide. *Appl. Anim. Behav. Sci.* **51**, 307–316.

26 Pournelle, G. H. 1965 Observations on the birth and early development of the spotted hyaena. *J. Mammal.* **46**, 503.

27 van Doorn, G.S., Hengeveld, G.M., Weissing, F.J. 2003 The evolution of social dominance I: Two-player models. *Behaviour* **140**, 1305-1332.

28 Hofer, H. & East, M.L. 1993a The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. I. Social organization. *Anim. Behav.* **46**, 547–557. (doi:10.1006/anbe.1993.1222)

29 Hofer, H. & East, M.L. 1993b The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. II. Intrusion pressure and commuters' space use. *Anim. Behav.* **46**, 559–574. (doi:10.1006/anbe.1993.1223)

30 Hofer, H. & East M.L. 2003 Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective. *Evol. Ecol.* **17**, 315–331. (doi: 10.1023/A:1027352517231)

31 Clutton-Brock, T.H. & Parker, G.A. 1995 Punishment in animal societies. *Nature* **373**, 209-216. (doi:10.1038/373209a0)

32 James,W.H. & Hofer, H. 1999 A note on sex ratios and sex combinations in Serengeti spotted hyaenas: siblicide and sub-binomial variance. *Appl. Anim. Behav. Sci.* **65**, 153-158.

33 Frank, L.G. 1986 Social organization of the spotted hyaena (*Crocuta crocuta*). I. Demography. *Anim. Behav.* **34**, 1500–1509. (doi:10.1016/S0003-3472(86)80221-4)

34 Altmann, J. 1974 Observational study of behaviour: sampling methods. *Behaviour* **49**, 227-267.

35 East, M.L., Burke T., Wilhelm, K., Greig, C. & Hofer, H. 2003 Sexual conflicts in spotted hyenas: male and female mating tactics and their reproductive outcome with respect to age, social status and tenure. *Proc. R. Soc. B* **270**, 1247-1254. (doi:10.1098/rspb.2003.2363.)

36 Parmar, M.K.B. & Machin D.1995 Survival analysis: a practical approach. John Wiley and Sons, Chichester

37 Bates, D. & Maechler, M. 2010 lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-35.

38 Burnham, K.P. and Anderson, D.R. 2002 *Model selection and multi-model interference: a practical information-theoretic approach*. Springer, New York.

39 Brunner, E.& Puri, M.L. 1996 Nonparametric methods in design and analysis of experiments. In: Ghosh,S. & Rao, C.R. (eds.) *Handbook of Statistics* **13**, 631-703, Elsevier Science B.V., Amsterdam.

40 Akritas, M.G. & Brunner, E. 1997 A unified approach to ranks tests in mixed models. *J. Stat. Planning Inf.* **61**, 249-277.

41 Madden, J.R, Hansjoerg, P.K, English, S., Manser, M.B. & Clutton-Brock, T.H. 2009 Calling in the gap: competition and cooperation in littermates' begging behaviour. *Proc. R. Soc. B* **276**, 1255-1262. (doi: 10.1098/rspb.2008.1660)

42 Trillmich, F. & Wolf, J.B.W. 2008 Parent-offspring and sibling conflict in Galapagos fur seals and sea lions. *Behav. Ecol. Sociobiol* **62**, 363 - 375.

43 Fawcett T.W and Johnstone R.A. 2010. Learning your own strength: winner and loser effects should change with age and experience. *Proc. R. Soc. B* **277**, 1427-1434. (doi:10.1098/rspb.2009.2088)

44 Bonisoli-Alquati, A., Giuseppe Boncoraglio, G., Caprioli, M. & Saino, N. 2011 Birth order, individual sex and sex of competitors determine the outcome of conflict among siblings over parental care. *Proc. R. Soc. B* **278**, 1273–1279. (doi:10.1098 / rspb.2010.1741)

45 Clutton-Brock, T.H. 1998 Reproductive skew and limited control. *Trends Ecol. Evol.* 1, 288-292.

Table 1 Summary of the best models with smallest Akaike Information Criterion (AIC) fitted to predict variation in (1) the relative delay between siblings (*rel. delay*), (2) the suckling time lost by the dominant (*domlat*) owing to its own aggression and (3) the suckling time lost by the subordinate sib owing to the aggression of the dominant sib (*sublat*). ΔAIC is the difference between the AIC of the intercept model and the AIC of the candidate model. All models included litter identity as a random effect.

selected	best models (with similar AIC (2 units))	AIC	ΔAIC
	relative delay between siblings (*rel. delay*)		
	mean.subscore, *age120*†	47.88	12.51
✓(model 1)	*mean.subscore*, *age120*†, *hunger*	49.82	10.57
	time lost suckling by the dominant (*domlat*)		
✓(model 2)	*sublat8, mean.domscore, age, mean.subscore(-1.7)*scomp2*‡	108.26	38.65
	time lost suckling by the subordinate (*sublat*)		
✓(model 3)	*domlat, mean.subscore, age130, hunger*mean.domscore*‡*, scomp2*domscore*‡	239.60	111.09
	domlat, *age130, hunger*mean.domscore*‡*,scomp2*domscore*‡	238.77	111.92

† *age120/130*: variable fitted as a piece-wise linear effect up to a threshold of 120 or 130 days, thereafter assuming no effect of the variable (see electronic supplementary material (ESM) 1 for details on assessing all piece-wise linear effects).

† *sublat8/mean.subscore(-1.7)*: variables fitted as a piece-wise linear effect up to a threshold at 8 seconds and -1.7, respectively, thereafter assuming no effect of the variable .

‡ *mean.subscore(-1.7)*scomp2*: interaction between both variables.

FIGURE CAPTIONS

Figure 1 The relative delay of both dominant and subordinate sibling in returning to suckling after aggression by the dominant interrupted suckling (*rel.delay*), in relation to (a) level of assertiveness of the subordinate (*mean.subscore*), (b) hunger level of the litter and (c) age (days), as predicted by model 1. The relative advantage of the dominant is higher when the relative delay tends towards 1, i.e. when the dominant returned much faster to suckle than the subordinate. Tick marks inside the x-axis indicate location of individual data points, dotted lines indicate confidence intervals. Unequal spacing on the y-axis is used to obtain straight lines (see ESM 2 for details on the methods used to produce figures 1 to 3).

Figure 2 Mean time (s) lost suckling by the dominant owing to its aggression (*domlat*) in relation to (a) time (s) lost suckling by the subordinate (*sublat*), (b) level of aggression of the dominant (*mean.domscore*), (c) age (days), (d) level of assertiveness of the subordinate (*mean.subscore*) and litter sex composition (*scomp2*) where ♀dom♀sub = all-female litter; ♂dom♂sub = all-male litter; ♀dom♂sub = mixed-sex litter with dominant female; ♂dom♀sub = mixed-sex litter with dominant male, as predicted by model 2. Tick marks on the x-axis indicate location of individual data points, dotted lines show confidence intervals.

Figure 3 The mean suckling time (s) lost by the subordinate owing to aggression by the dominant (*sublat*) in relation to (a) suckling time (s) lost by the dominant (*domlat*), (b) level of assertiveness of the subordinate (*mean.subscore*), (c) age (days), (d) level of aggressiveness of the dominant (*mean.domscore*) and hunger level of the litter, (e) level of aggressiveness of the dominant and litter sex composition (*scomp2*) where ♀dom♀sub = all-female litter; ♂dom♂sub = all-male litter; ♀dom♂sub = mixed-sex litter with dominant female; ♂dom♀sub = mixed-sex litter with dominant male, as predicted by model 3. Tick marks on the x-axis indicate the location of individual data points, dotted lines show confidence intervals.

Figure 4 Short-term change in the submissive response of the subordinate to repeated aggression by the dominant per focal sample in relation to age (days). Change in behaviour was expressed as the slope of the regression of *subscore* over consecutive interactions against time. Highly negative values indicate a strong loser effect; values close to zero indicate little change in response. The three most negative values represent three different litters. The vertical dotted line illustrates the change in tendencies after 90 days of age, the mean age before which siblicide occurs [4,25].

Figure 1

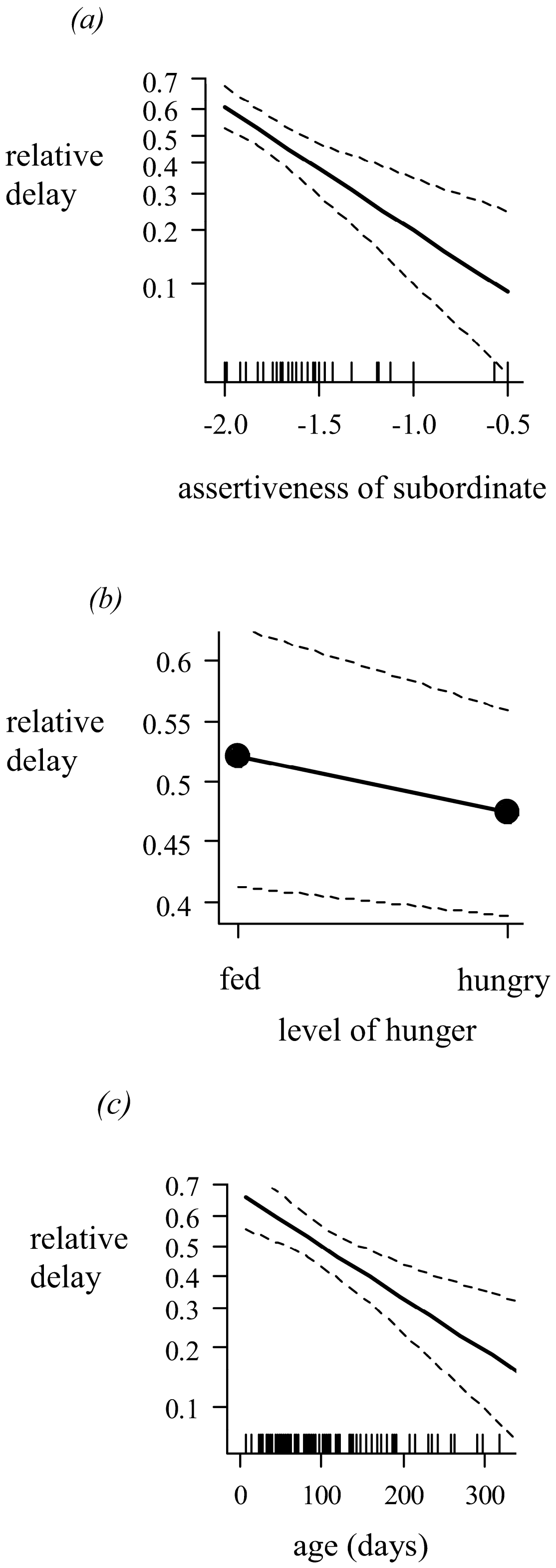

(a)
relative delay
0.7
0.6
0.5
0.4
0.3
0.2
0.1
-2.0
-1.5
-1.0
-0.5
assertiveness of subordinate
(b)
relative delay
0.6
0.55
0.5
0.45
0.4
fed
hungry
level of hunger
(c)
relative delay
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
300
age (days)

Figure 2

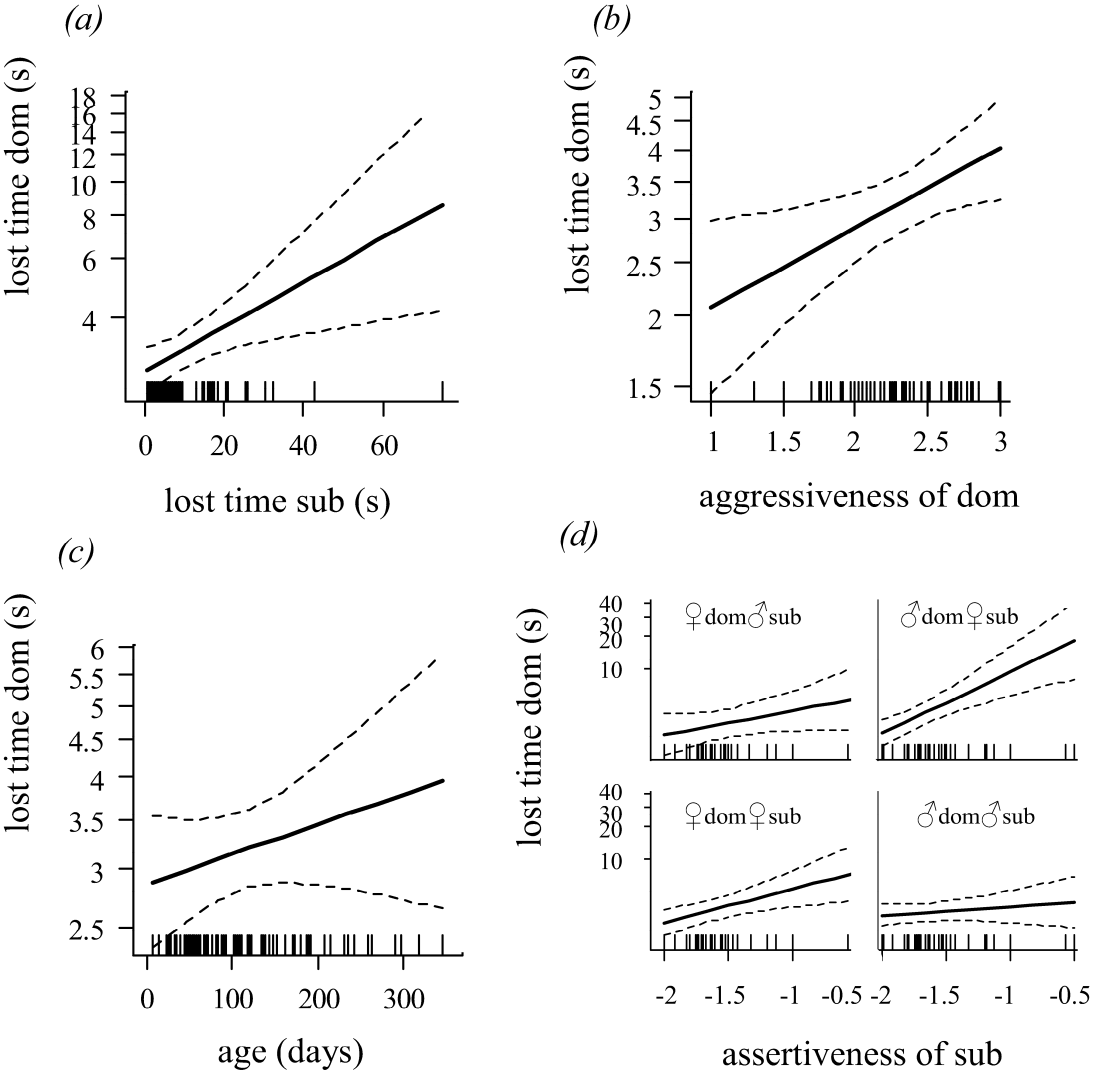

(a)
lost time dom (s)
18
16
14
12
10
8
6
4
0
20
40
60
lost time sub (s)
(b)
lost time dom (s)
5
4.5
4
3.5
3
2.5
2
1.5
1
1.5
2
2.5
3
aggressiveness of dom
(c)
lost time dom (s)
6
5.5
5
4.5
4
3.5
3
2.5
0
100
200
300
age (days)
(d)
lost time dom (s)
40
30
20
10
♀dom♂sub
♂dom♀sub
♀dom♀sub
♂dom♂sub
-2
-1.5
-1
-0.5
assertiveness of sub

Figure 3

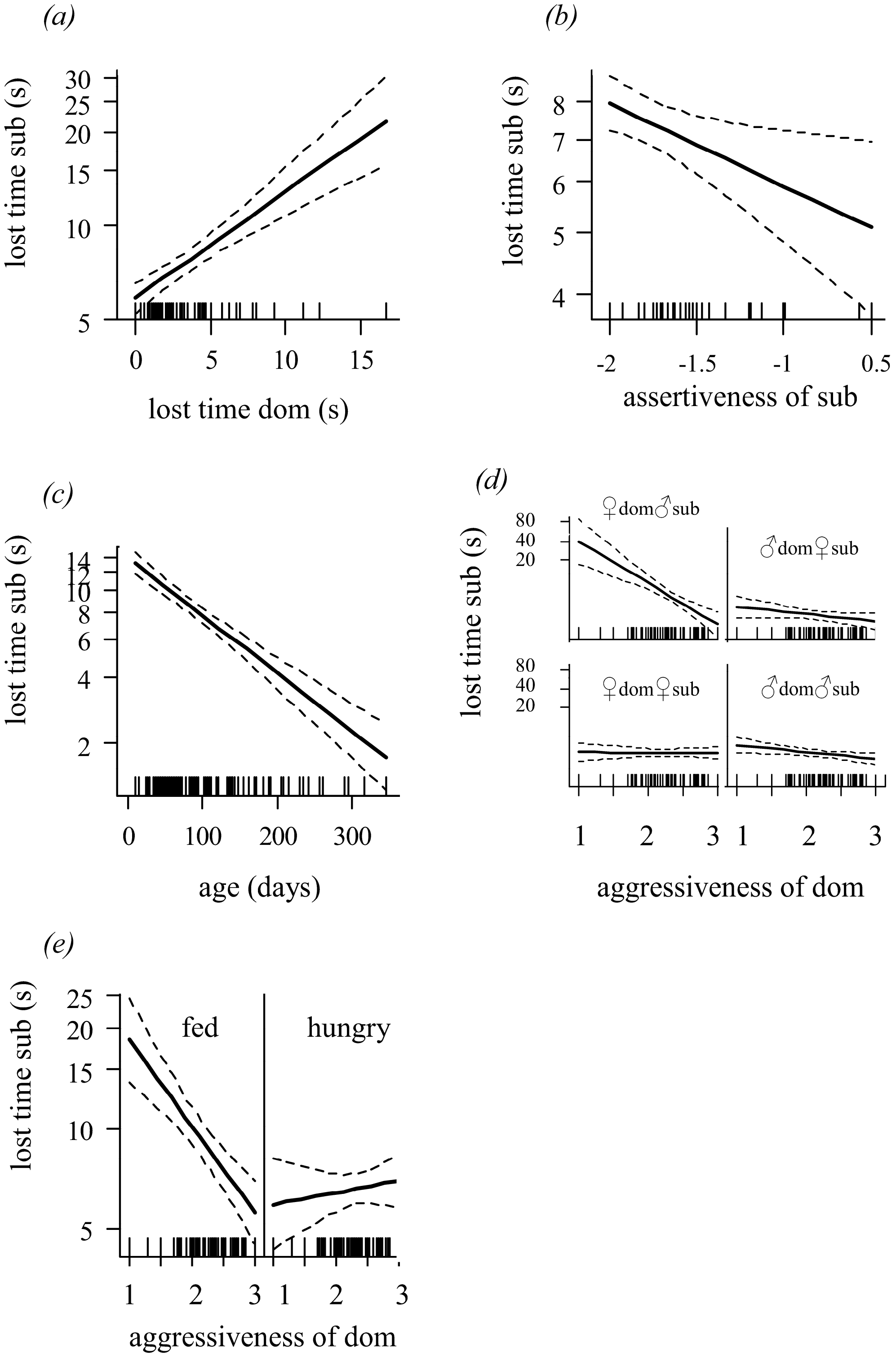
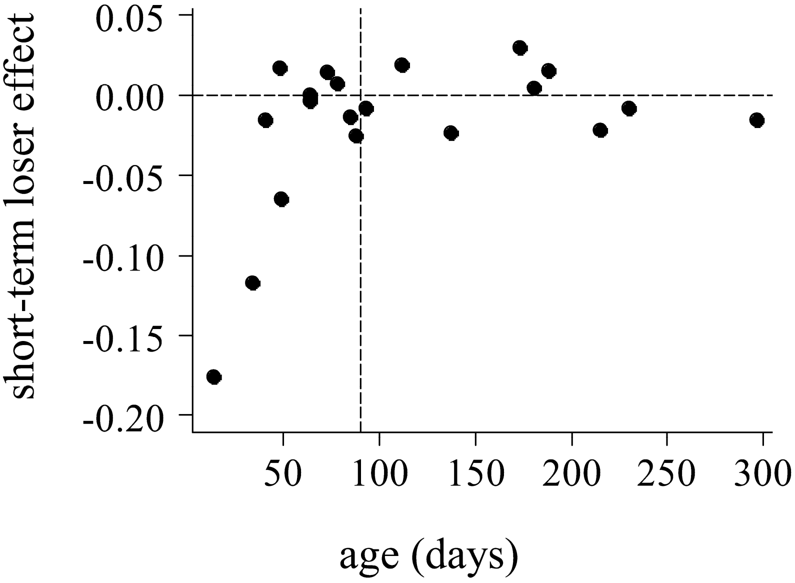

(a)
lost time sub (s)
30
25
20
15
10
5
0
5
10
15
lost time dom (s)
(b)
lost time sub (s)
8
7
6
5
4
-2
-1.5
-1
0.5
assertiveness of sub
(c)
lost time sub (s)
14
12
10
8
6
4
2
0
100
200
300
age (days)
(d)
lost time sub (s)
♀dom♂sub
♂dom♀sub
♀dom♀sub
♂dom♂sub
80
40
20
80
40
20
1
2
3
1
2
3
aggressiveness of dom
(e)
lost time sub (s)
25
20
15
10
5
fed
hungry
1
2
3
1
2
3
aggressiveness of dom

Figure 4


short-term loser effect
0.05
0.00
-0.05
-0.10
-0.15
-0.20
50
100
150
200
250
300
age (days)

Electronic supplementary material 1 (ESM 1): detailed methods

'Sibling rivalry: training effects, emergence of dominance and incomplete control'

Sarah Benhaiem [1], Heribert Hofer [1], Stephanie Kramer-Schadt [1], Edgar Brunner [2], Marion L. East [1]

[1] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

[2] University of Göttingen, Faculty of Medicine, Department of Medical Statistics, Humboldtallee 32, 37073 Göttingen, Germany

This document contains supporting information on the study species, and all procedures and data used to estimate independent and dependent variables. References to the literature are cited and listed in the original article, except for references [46-59] listed at the end of this document.

Study site

In our study site, territories of clans experienced large fluctuations in the density of migratory herbivores including wildebeest *Connochaetes taurinus*, Thomson's gazelle *Gazella thomsoni*, and plains zebra *Equus burchelli* [28, 29]. We monitored cub behaviour at communal or private birth dens [46]. Adult hyenas are too large to enter dens beyond the entrance, thus cubs of all ages, including newborn cubs, emerge from the den entrance to suckle [4, 22].

Demographic variables

Cub age was estimated (*age*, ±7 days) using pelage characteristics, the degree to which ears were extended plus the degree of coordination [26]. Cubs were sexed using the dimorphic glans morphology of the erect phallus [47] at the age of ca. 3 months.

Behavioural data

In all 97 recorded focal samples, at least one agonistic interaction occurred. One observer analysed all videos using the specialist behavioural software INTERACT (Mangold, Straubing, Germany). Females normally only suckle their own offspring [19, 30] although rare cases of adoption by surrogate mothers occur [48]. In this data set one focal sample came from a litter suckled by a surrogate mother. Following [22] we classified a cub as the subordinate member of the litter if the number of submissive behaviours observed for that cub was greater than that recorded for its littermate. We observed clear dominant and subordinate roles. In one litter a change in dominance status occurred between two successive focal samples. As dominant and subordinates roles were inverted after the change in dominance, we treated data obtained from before and after the dominance change separately.

Do birth order, body mass at birth and sexual dimorphism influence the emergence of within-litter dominance relationships?

The interval between the birth of twin spotted hyenas might create a competitive asymmetry to help the first born dominate the second born, but currently it is unknown whether birth order shapes the dominance relationship between spotted hyena littermates [49,50]. Births of litters in the wild are very rarely observed and birth order in twin litters was not known in this study. Frank, Glickman and Licht [49] reported video observations on 5 captive twin litters of spotted hyenas. Neonates were delivered about one hour apart, and the first born usually attacked the second within minutes of birth. The second born fought back quickly and did not accept a subordinate role. The authors did not mention whether the first born cub in any of these litters eventually became the dominant, as they only noted that “one sibling” established dominance over the other in the first days. Similarly, Smale et al. [50] did not determine whether the first born of 9 captive litters became the dominant sibling. Thus, even in captive litters it was unclear whether first born cubs are more likely to become dominants than second born cubs.

Smale et al. [50] provided information on weight in seven captive twin litters, and suggested that initially heavier siblings tended to become dominants, because in 5 of 7 twin litters, dominants weighted (mean ± s.e.m.) 8.8 ± 1.8% more than subordinates (no statistical test provided). However, members of twin litters could only be individually identified and weighted at ages ranging from 1 hour to 8 days after birth (mean age at which cubs were individually identified and weighted: 2.8 ± 3 days, median = 2 days, see their Table III p. 679), leaving it unclear whether the disparity in weight existed at birth or was the consequence of biasing milk supply in favour of one sibling after birth. In one mixed-sex twin litter, on day 3 the dominant sib was female and 7.3 % heavier, in another mixed-sex twin litter on day 2 the dominant was male and 6.2 % heavier. It therefore remains unclear whether (1) there is a weight disparity between males and females at birth, and (2) whether females (or males) are always born first (or second). Smale et al. [50] do not report birth weights, but weights after at least 1 hour (in 3 litters) or 24 hours (in 6 litters) of sibling rivalry and milk ingestion. We conclude that there is currently insufficient evidence to support the idea that there is a clear weight disparity at birth or that any weight disparity may be influenced by sibling sex.

Monopolisation of both maternal teats by the dominant sibling

The dominant sibling suckles in a preferred position along the mother's belly, thereby facilitating monopolising access to both maternal teats. The subordinate sibling suckles most often from a posterior position between the mother's hind legs, or by stretching over her rump or lying parallel to but beneath the dominant cub: in all three positions its access to maternal teats is more restricted than that of the dominant sibling (supplementary figure 1).

Supplementary figure 1: Sibling conflict during a suckling bout. Top: The dominant (right) has stopped suckling and glares (threatens) its subordinate sibling (left) which has retreated with its ears back. Bottom: the dominant suckles and the subordinate sibling attempts to suckle (photos: Sarah Benhaiem).

Predictor variables

Maternal standardised rank. Dominance hierarchies in each clan were adjusted after each loss or recruitment of adult females.

Prey abundance in the territory. Migratory herbivores were responsible for large fluctuations in prey abundance within clan territories [28, 29] which profoundly affect maternal foraging distances and foraging success and thus their opportunity to provide dependent cubs with milk [19, 29]. Changes between these levels of abundance occurred repeatedly throughout the year, thereby influencing the extent of long-distance foraging and the level of maternal input [4, 19, 25, 29, 30].

Maternal presence at the communal den. Mothers with dependent cubs that return to the communal den after a foraging trip always suckle their litter [19,22,25], thus the presence of a mother at the den is strong evidence that cubs were nursed. This was repeatedly verified during the many years when litters were regularly weighed in our study clans.

These three predictors (maternal standardised rank, prey abundance, maternal presence at the den) significantly affect offspring cub growth rate and thus are reliable predictors for suckling in terms of maternal input. Hofer and East [19] demonstrated that cub growth rates increase with the proportion of visits to the den by the mother (figure 5 in [19]) (i.e. our variable maternal attendance), maternal absence intervals and prey abundance in the territory (figure 6 in [19]). Hofer and East [30] also demonstrated that early cub growth (within the first 6 months of life) increases with increasing maternal social status (figure 2 in [30]). Cub growth rate was affected by maternal social status and prey abundance, because maternal social status determines priority of access to food within the territory, as previously described.

Hunger level. This variable is a general measure that combines information on prey abundance in the territory and information on maternal presence at the communal den. Because prey abundance and maternal presence at the clan den significantly affect cub growth rates, they influence cub suckling and their relative “hunger” levels:

1) Cubs were considered “fed” when the mother visited the den (i.e. during the 30 hours prior to a focal sample). Cub body mass increased when the mother was present at the den and declined during a mother’s absence from the den, regardless of whether we actually witnessed the nursing period. The only exception to this were cases of siblicide when dominant cubs entirely excluded their subordinate littermate from nursing after the return of the mother, but there were no such cases in our current study.

2) Cubs were considered “fed” when prey abundance was high. When prey abundance was “high” in a clan territory, mothers “over-feed” (i.e., female hyenas consumed up to 18 kg of food), digestive processes caused spotted hyenas to over-heat and spend daylight hours digesting in cool locations – and thus may return to the den well after dark, when the heat of the day had passed, possibly after our “dusk observation periods” which ended at about 20.00hrs. We are certain that during periods of high prey abundance, no lactating female travelled outside the clan territory to feed, and all our experience of observing nursing patterns at communal dens leaves us in very little doubt that the two females not seen in the **2 (of 11) focal samples** recorded during periods of high prey availability returned to den and suckled their cubs within a 30h period. In **37** out of **39 focal samples** in the category “fed” the mother was present at the den and suckled her cubs in a period of 30 hours prior to the focal sample.

In addition to both categories, as a rough confirmation of their validity, the same observer recorded the general “state” of the siblings and attributed it to one of the following categories: “well fed”, “normal” or ”thin”, which were generally consistent with our categories of hunger level.

Sex ratio and mixed-sex litters. The binomial test used to investigate whether females are more often dominant than males in mixed-sex litters was based on the assumption of a 0.5 sex ratio. Hofer and East [25] and James and Hofer [32] demonstrated that there was no sex ratio bias evident in this species at 3 months of age. This overall result masked an excess of females in singleton litters and an excess of males in twin litters. They proposed and tested three hypotheses that would explain the sex ratio biases they observed: (1) the primary sex ratio was skewed, (2) cub mortality was female biased, or (3) the biases were a consequence of siblicide. A shared consequence of the first two hypotheses would have been a same-sex bias both within singletons and within twin litters. This was not the case, supporting the idea that there was no bias in the primary sex ratio. The results were consistent with the third hypothesis and suggested that the lack of all-female twin litters and the predominance of singleton females were a consequence of a higher incidence of siblicide in all-female twin litters. Later, James and Hofer [32] developed a model to further test this hypothesis and demonstrated that the probability of siblicide depended on the sex of both the dominant and the subordinate sib and that the occurrence of facultative siblicide was more frequent in all-female litters. Wahaj et al. [51] also reported a similar sex ratio for a clan a clan in Kenya of 0.522

(140 males, 128 females, non significantly biased towards one sex) at 2 - 3 months of age and a sex ratio of 0.512 in captivity (21 males, 20 females).

Litter sex composition. Litters consisted of 21 same-sex cubs (*scomp1*=1; for parameter abbreviations see supplementary table 1), either two female cubs (*scomp2*=1; 9 litters) or two male cubs (*scomp2*=2; 12 litters) and of 17 mixed-sex (*scomp1*=2) litters. In mixed-sexed litters, the dominant sibling was female and the subordinate male (*scomp2*=3; 7 litters) or the dominant male and the subordinate female (*scomp2*=4; 10 litters).

Sibling tactics. The intensity of the dominant's aggression was measured using the following categories: (1) low intensity aggression: glaring at the sibling; (2) moderate intensity aggression: pushing, mouth-wrestling, blocking access to the mother, nipple-switching and supplanting the sibling from one nipple; (3) high intensity aggression: lunging, biting, bite-shaking, chasing.

The subordinate's behavioural response to the dominant's aggression was classified as follows: (-2) highly submissive: retreats or leaves with ears back and submissive posture; (-1) marginally submissive: only ears back; (0) no response; (1) marginally aggressive: glaring at the sibling; (2) moderately aggressive: pushing, mouth-wrestling, blocking access to the mother, nipple-switching and supplanting the sibling from one nipple (supplementary table 2).

As disturbance of litters by other clan members during suckling bouts increased within-litter aggression [22], we controlled for disturbances of suckling litters by other clan members or indirectly by aggressive acts that forced mothers to move by excluding all focal samples or agonistic interactions in which interference or disturbance occurred (N=8).

Loser effect. We used a minimum of 17 agonistic interactions to test for loser effects because this provided sufficient power for the regressions of *subscore* values against time elapsed during focal samples and we assumed that documenting a learning effect well presupposed a minimum number of interactions and a well expressed change in the subordinate's response to the dominant's aggression.

Statistical analysis

To assess which variables were most likely to influence the response variable and to check for correlations between variables we used Kendall's τ. The variables *sublat* and *age* were significantly positively correlated (N = 97 focal samples, P <0.01) and the variables *domlat* and *mean.subscore* were also significantly positively correlated (N = 97 focal samples, P < 0.01).

As in both cases the strength of the correlation was low (τ = - 0.33 and τ = 0.33) we included both *sublat* and *age* together and *domlat* and *mean.subscore* together in the three sets of candidate models.

The Akaike Information Criterion (AIC) measures the best compromise between accuracy and precision. The information theoretic approach accepts that uncertainty is an inherent feature of biological data and focuses on the plausibility of different hypotheses [52]. As such model selection allows the simultaneous evaluation of multiple hypotheses, it is especially suited for observational studies in behavioural ecology [52,53]. This method does not attempt to measure the probability of seeing data given an assumed model, nor does it employ arbitrary cut-offs to decide which estimates are “significant”, thus specific *p*-values for individual predictor variables are not provided [54].

Generalised Additive Models (GAM)

We used Generalised Additive Models (GAMs, gam package of R [55]) to assess the shape of the relationship between each independent continuous variable and the dependent variables (1) *rel. delay*, (2) *domlat* and (3) *sublat* [56, 57]. Candidate models were fitted with polynomial functions, linear, or piece-wise linear responses of fixed effects in accordance with the results from GAM models. When using GAMs we fitted smoothing splines with 3 degrees of freedom and transformed the non-linear variables into suitable parametric terms after graphical inspection of the partial residual plots [58].

The visual inspection of GAMs for (1) the relative delay (*rel. delay)* indicated a non-linear relationship for litter age (*age*). The predictor *age* was modelled as a piece-wise linear effect. The best threshold according to the lowest residual deviance was 120 days for the age of siblings, beyond which no effect of age on *rel. delay* was assumed.

The inspection of GAMs for (2) the time lost suckling by the dominant (*domlat)* indicated a non-linear relationship for the subordinate’s time lost suckling *(sublat)* and for the subordinate’s intensity of submission *(mean.subscore)*. The predictors *sublat* and *mean.subscore* were modelled as piece-wise linear effects with thresholds at 8 seconds and -1.7, respectively, beyond which no effects of *sublat* and *mean.subscore* on *domlat* were assumed.

The inspection of GAMs for (3) the time lost suckling by the subordinate (*sublat*) indicated a non-linear relationship for litter age (*age)* and for maternal social status *(mstatus).* The predictors *age* and *mstatus* were modelled as piece-wise linear effects with a threshold at 130 days for litter age and at 0.1 for *mstatus*, beyond which no effect of *age* and *mstatus* on *sublat* were assumed.

Generalised Linear Mixed Models (GLMM)

Three sets of candidate GLMM models were fitted with linear or piece-wise linear responses of fixed effects in accordance with the GAM results. The candidate models corresponded to different biological hypotheses: the response variable (*rel. delay / domlat / sublat*) is influenced by (1) sibling behaviours i.e. sibling times lost suckling (*domlat*, *sublat*) or sibling tactics (*mean.domscore* and/or *mean.subscore*); (2) the level of hunger of siblings estimated with *mstatus*, *prey* and *hunger* (3) demography, i.e.*, age, dsex, ssex, scomp1* or *scomp2* (see supplementary table 1) and (4) a combination of the most influential uncorrelated behavioural, ecological and demographic variables, including the most influential interactions. Parameters were estimated by maximum likelihood using the Laplace approximation to evaluate the marginal likelihood [59].

We modelled (1) the relative delay (*rel. delay*) as a quasibinomial distribution (logit link), since dominant and subordinate mean latencies as input parameters for *rel. delay* were not binomial data such as “success” or “failure” but had been used to create a standardised proportion. We rounded the time lost by the dominant (2) *domlat* and by the subordinate (3) *sublat* to obtain counts of seconds when they were used as response variables and modelled them as poisson distributions (log link). We excluded from this analysis the cases (5 from 97 focal samples) where the subordinate did not return to suckling after an agonistic interaction (*sublat* = minimum of 900 seconds).

Supplementary table 1 Description and abbreviation of variables recorded and used in data analysis

variables	**abbreviation**	**level**	**values range**
predictors / independent variables			
maternal social status	*mstatus*	continuous	- 1 to + 1
prey abundance (1=low, 2=medium, 3=high)	*prey*	categorical	1,2 or 3
level of hunger (1= fed, 2=hungry)	*hunger*	categorical	1 or 2
age of siblings (days)	*age*	continuous	9 to 346
dominant sex (1=male, 2=female)	*dsex*	categorical	1 or 2
subordinate sex (1=male, 2=female)	*ssex*	categorical	1 or 2
sex composition of the litter 1 (1=same-sex litter, 2=mixed-sex litter)	*scomp1*	categorical	1 or 2
sex composition of the litter 2 (1=female female,2=male male,3=female dominant, male sub, 4=male dominant, female sub)	*scomp2*	categorical	1,2,3 or 4
mean intensity of dominant's aggression	*mean.domscore*	continuous	+1 to +3
mean intensity of subordinate's submission/assertiveness	*mean.subscore*	continuous	- 2 to + 3
dependent variables			
relative delay between siblings	*rel.delay*	continuous	0 to + 1
dominant's mean latency [s] to return to suckling after it is aggressive	*domlat*	continuous	0 to +16.60
subordinate's mean latency [s] to return to suckling after the dominant is aggressive	*sublat*	continuous	0.25 to min. 900

Supplementary table 2 Aggressive and submissive behaviours of dominant and subordinate sibs and their transformation into individual *domscore* and *subscore* values to indicate the intensity of the dominant's aggression and the subordinate's submission, respectively.

behaviours	**levels of aggression and submission**	***domscore, subscore***
retreating with ears back; leaving with ears back	high level of submission	- 2
ears back	moderate level of submission	- 1
none	no response to aggression	0
glaring	low level of aggression	+ 1
pushing; blocking the access to teat; mouth-wrestling; supplanting sib at teat	moderate level of aggression	+ 2
lunging; biting; bite-shaking; chasing	high level of aggression	+ 3

REFERENCES

46 East, M.L., Hofer, H. & Türk, A. 1989 Functions of birth dens in spotted hyaenas (*Crocuta crocuta*). *J. Zool. (Lond.)* **219**, 690-697. (doi:10.1111/j.1469-7998.1989.tb02614.x)

47 Frank, L.G., Glickman, S.E. & Powch, I. 1990 Sexual dimorphism in the spotted hyaena (*Crocuta crocuta*). *J. Zool. (Lond.)* **221**, 308-313. (doi:10.1111/j.1469-7998.1990.tb04001.x)

48 East, M.L., Höner, O.P., Wachter, B., Wilhelm, K., Burke, T. & Hofer, H. 2009 Maternal effects on offspring social status in spotted hyenas. *Behav. Ecol.* **20**, 478-483. (doi:10.1093/beheco/arp020)

49 Frank, L.G., Glickman, S.E. & Licht, P. 1991 Fatal sibling aggression, precocial development, and androgens in neonatal spotted hyenas. *Science.* **252**, 702-704.

50 Smale, L., Holekamp, K.E., Weldele, M., Frank, L.G. & Glickman S.E. 1995 Competition & cooperation between littermates in the spotted hyaena *(Crocuta crocuta*). Anim. Behav. **50**, 671-682.

51 Wahaj, S.A., Place, N.J., Weldele, M.L., Glickman, S.E. & Holekamp, K.E. 2007 Siblicide in the spotted hyena: analysis with ultrasonic examination of wild and captive individuals. Behav. Ecol. **18**, 974–984. (doi:10.1093/beheco/arm076)

52 Garamszegi, L.Z. 2011 Information-theoretic approaches to statistical analysis in behavioural ecology: an introduction. *Behav. Ecol. Sociobiol.* **65**, 1-11. (doi:10.1007/s00265-010-1028-7)

53 Johnson, J.B. & Omland, K.S. 2004 Model selection in ecology and evolution. *Trends Ecol. Evol.* **19**, 101-108. (doi:10.1016f/j.tree.2003.10.013)

54 Beheim, B.A. & Bell, A.V. 2011 Inheritance, ecology and the evolution of the canoes of east Oceania. *Proc. R. Soc. B*, in press

55 Hastie, T. 2010 gam: generalized additive models. R package version 1.03. http://CRAN.R-project.org/package=gam

56 Klar, N., Fernandez, N.F., Kramer-Schadt, S., Herrmann, M., Trinzen, M., Büttner, I. & Niemitz, C. 2008 Habitat selection models for European wildcat conservation. *Biol. Conserv.* **141**, 308-319. (doi:10.1016/j.biocon.2007.10.004)

57 Zuur, A.F., Ieno, E.N. & Elphick, C.S. 2010 A protocol for data exploration to avoid common statistical problems. *Methods Ecol. Evol.* **1**, 3-14. (doi: 10.1111/j.2041-210X.2009.00001.x)

58 Crawley, M.J. 2005 *Statistics: An introduction using R.* JohnWiley & Sons Ltd., Chichester.

59 Skaug, H. & Fournier, D. 2005 Automatic evaluation of the marginal likelihood in nonlinear hierarchical models. Unpublished, available from: http://bemata.imr.no/laplace.pdf

Electronic supplementary material 2 (ESM 2): additional results

‘Sibling rivalry: training effects, emergence of dominance and incomplete control’

Sarah Benhaiem [1], Heribert Hofer [1], Stephanie Kramer-Schadt [1], Edgar Brunner [2], Marion L. East [1]

[1] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

[2] University of Göttingen, Faculty of Medicine, Department of Medical Statistics, Humboldtallee 32, 37073 Göttingen, Germany

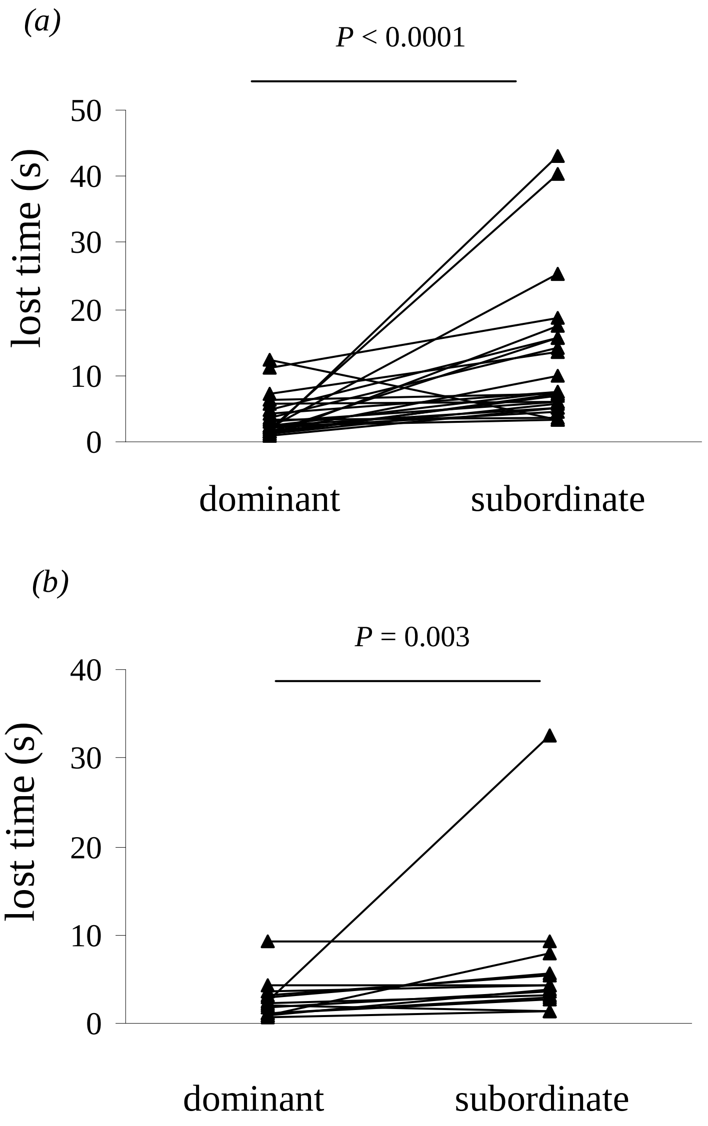
This document contains information on the methods (“effect plots”) used to produce figures 1, 2 and 3 and additional results.

Information on effect plots (package “effects” in R) used to produce figures 1 to 3 in the article, and supplementary figures 4 to 6 in ESM3.

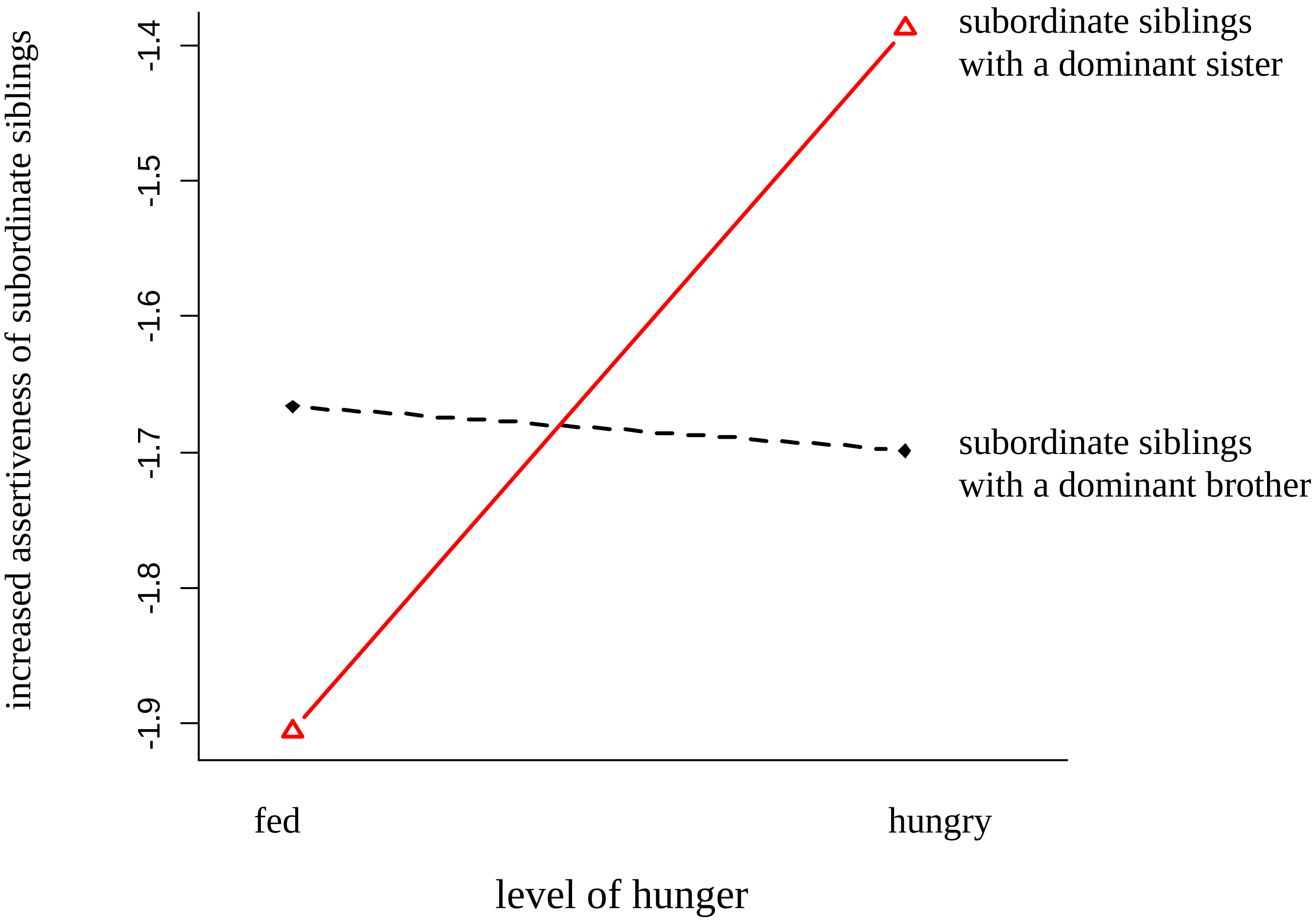
This method, developed by [60], permits the graphical display of the effective contribution of different parameters in a generalised linear model in an appropriate way. The standard and default option is to plot the effects of the predictors on the scale of the linear predictor (logit [“model 1”, figure 2, supplementary figure 4 in ESM3] and log [“model 2” and “model 3”, figure 3 and supplementary figures 5 and 6 in ESM 3]) but to label the vertical axis on the scale of the response. This option enables to obtain straight lines and results in an unequal spacing of the tick marks on the y-axis.

[60] Fox, J. 2003 Effect displays in R for generalised linear models. *J. Stat. Softw.*, **8** (15), 1-27.

Supplementary figure 2. Mean period (s) between the cessation of suckling by the dominant (*domlat*, left) or the subordinate member (*sublat*, right) of a twin litter after an aggressive act by the dominant, and the resumption of suckling by both siblings. (*a*) Twenty-four litters less than 120 days of age. (*b*) Fourteen litters more than 120 days of age. Five focal samples in which the subordinate did not return to suckling (equivalent to a lost time of a minimum of 900s) after an aggressive act by the dominant are not shown on this figure.



Supplementary figure 3. Mean level of assertiveness of the subordinate sibling in response to aggression by the dominant, as a function of the interaction between the sex of the dominant sibling (*dsex*) and the hunger level of the litter (*hunger*). The values on the y-axis are the mean value of all *subscore* values recorded for one litter in one category of hunger (see methods in article for more details).



Supplementary table 3 Estimated coefficients and standard errors for the variables of chosen models for (1) *rel. delay*, (2) the lost suckling time (s) of the dominant sib *(domlat)* and (3) the lost suckling time (s) of the subordinate sib (*sublat)*. Explanations for abbreviated variable names are provided in caption of table 1 (article) and supplementary table 1 in electronic supplementary material 1.

	variables	**estimates of regression coefficients**
model 1	Intercept	– 3.76 ± 0. 38
	mean.subscore	– 1.81 ± 0.19
	hunger = 2	– 0.11 ± 0.12
	age120	– 0.02 ± 0.002
model 2	Intercept	0.76 ± 0.33
	sublat8	0.20 ± 0.03
	mean.domscore	0.28 ± 0.13
	age	0.002 ± 0.0008
	mean.subscore(-1.7) scomp2=♂♂*	0.22 ± 0.25
	mean.subscore(-1.7) scomp2=♀♂*	0.15 ± 0.27
	mean.subscore(-1.7) scomp2=♂♀*	0.95 ± 0.31
model 3	Intercept	1.99 ± 0.53
	domlat	0.12 ± 0.02
	mean.subscore	– 0.18 ± 0.17
	*mean.domscore*hunger=2*	0.48 ± 0.20
	age130	– 0.01 ± 0.002
	*mean.domscore1*scomp2=♂♂*	0.08 ± 0.24
	*mean.domscore1*scomp2=♀♂*	– 2.01 ± 0. 05
	*mean.domscore1*scomp2=♂♀*	– 0.07 ± 0.27

Electronic supplementary material 3 (ESM 3): additional analyses

‘Sibling rivalry: training effects, emergence of dominance and incomplete control’

Sarah Benhaiem [1], Heribert Hofer [1], Stephanie Kramer-Schadt [1], Edgar Brunner [2], Marion L. East [1]

[1] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

[2] University of Göttingen, Faculty of Medicine, Department of Medical Statistics, Humboldtallee 32, 37073 Göttingen, Germany

This document contains the results of an additional analysis of the best predictors influencing (1) the relative delay, (2) the time lost suckling by the dominant (seconds), (3) the time lost suckling by the subordinate (seconds) and (4) short-term training effects. In this analysis we investigate the effects of a single variable (named “*mean.score*”) which combines the effects of both dominant and subordinate sibling behaviours and which measures the level of dominance exerted by the dominant on the subordinate. This variable replaces the use of both variables measuring (1) the aggressiveness of the dominant (*mean.domscore*, see article) and (2) the assertiveness of the subordinate (*mean.subscore,* see article) sibling.

METHODS

Predictor variables

Sibling tactics

For each agonistic interaction we added up the dominant’s score to the subordinate’s score to obtain a score for one agonistic interaction (e.g. +1 (a low intensity aggression by the dominant) plus -2 (a highly submissive response by the subordinate) yields *score* of -1 for the interaction; supplementary table 2 in ESM 1). We calculated for each focal sample the mean score for all agonistic interactions (*mean. score*). A low *mean.score* (below 0) indicated that the dominant generally employed a low level of aggressive behaviour during the focal sample and that the subordinate responded with highly submissive behaviours (high level of dominance). A high *mean.score* (above 1) indicated that the response of the subordinate was not always submissive. The higher the value of *mean.score*, the less submissive were the responses of the subordinate; therefore when *mean.score* increased, the level of dominance exerted by the dominant sibling diminished.

Training effects

We tested for training effects using focal samples with a minimum of 17 interactions (20 focal samples, 14 litters, see ESM 1 for more details) by studying the sequence of values throughout a focal sample which describe the intensity of the dominant's aggression and the subordinate's response (*score*, the value of one agonistic interaction, ESM 1). An increase in the subordinate's submissive response in the course of a focal sample would produce a significant decrease in the *score* values and thus indicate a (short-term) training effect. Using the significant slopes of linear regressions of *score* plotted against time elapsed during the focal sample we tested whether the regression slopes increased with litter age (after logarithmic transformation of litter age (base 10) to obtain a linear relationship), i.e., that training effects were particularly conspicuous at an early age and diminished at later ages. The residuals of this linear regression model were normally distributed (Lilliefors tests) and variances did not show evidence of heterogeneity (residual plots).

Statistical analysis

To assess which variables were most likely to influence the response variable and to check for correlations between variables we used Kendall's τ. The variables *domlat* and *mean.score* were significantly positively correlated (N = 97 focal samples, $P < 0.01$). As the strength of the correlation was low ($\tau = 0.27$) we included both *domlat* and *mean.subscore* together in the three sets of candidate models that were compared with each other.

Generalised Additive Models (GAM)

We used Generalised Additive Models (GAMs, gam package of R [55]) to assess the shape of the relationship between each independent continuous variable and the dependent variables [56,57]. Candidate models were fitted with polynomial functions, linear, or piece-wise linear responses of fixed effects in accordance with the results from GAM models. When using GAMs we fitted smoothing splines with 3 degrees of freedom and transformed the non-linear variables into suitable parametric terms after graphical inspection of the partial residual plots [58].

The visual inspection of GAMs for (1) the relative delay (*rel. delay)* indicated a non-linear relationship for litter age (*age).* The predictor *age* was modelled as a piece-wise linear effect. The best threshold according to the lowest residual deviance was 120 days for the age of siblings, beyond which no effect of age on *rel. delay* was assumed.

The inspection of GAMs for (2) the time lost suckling by the dominant (*domlat)* indicated a non-linear relationship for the subordinate's time lost suckling *(sublat)* and for age. The predictors *sublat* and *age* were modelled as piece-wise linear effects with thresholds at 9 seconds and 180 days, respectively, beyond which no effects of *sublat* and *age* on *domlat* were assumed.

The inspection of GAMs for (3) the time lost suckling by the subordinate (*sublat*) indicated a non-linear relationship for litter age (*age)* and for maternal social status *(mstatus).* The predictors *age* and *mstatus* were modelled as piece-wise linear effects with a threshold at 100 days for litter age and a threshold at 0.2 for maternal social status, beyond which no effect of *age* and *mstatus* on *sublat* was assumed.

Generalised Linear Mixed Models (GLMM)

These transformed variables were included in three sets of candidate models. The candidate models were fitted with linear or piece-wise linear responses of fixed effects in accordance with the GAM results. The candidate models corresponded to different biological hypotheses: the response variable (*rel. delay / domlat / sublat*) is influenced by (1) sibling behaviours i.e. sibling times lost suckling (*domlat*, *sublat*) or sibling tactics (*mean.domscore* and/or *mean.subscore*); (2) the level of hunger of siblings estimated with *mstatus*, *prey* and *hunger* (3) demography i.e. *age, dsex, ssex, scomp1* or *scomp2* (see supplementary table 1) and (4) a combination of the most influential uncorrelated behavioural, ecological and demographic variables, including the most influential interactions. Parameters were estimated by maximum likelihood using the Laplace approximation to evaluate the marginal likelihood [59].

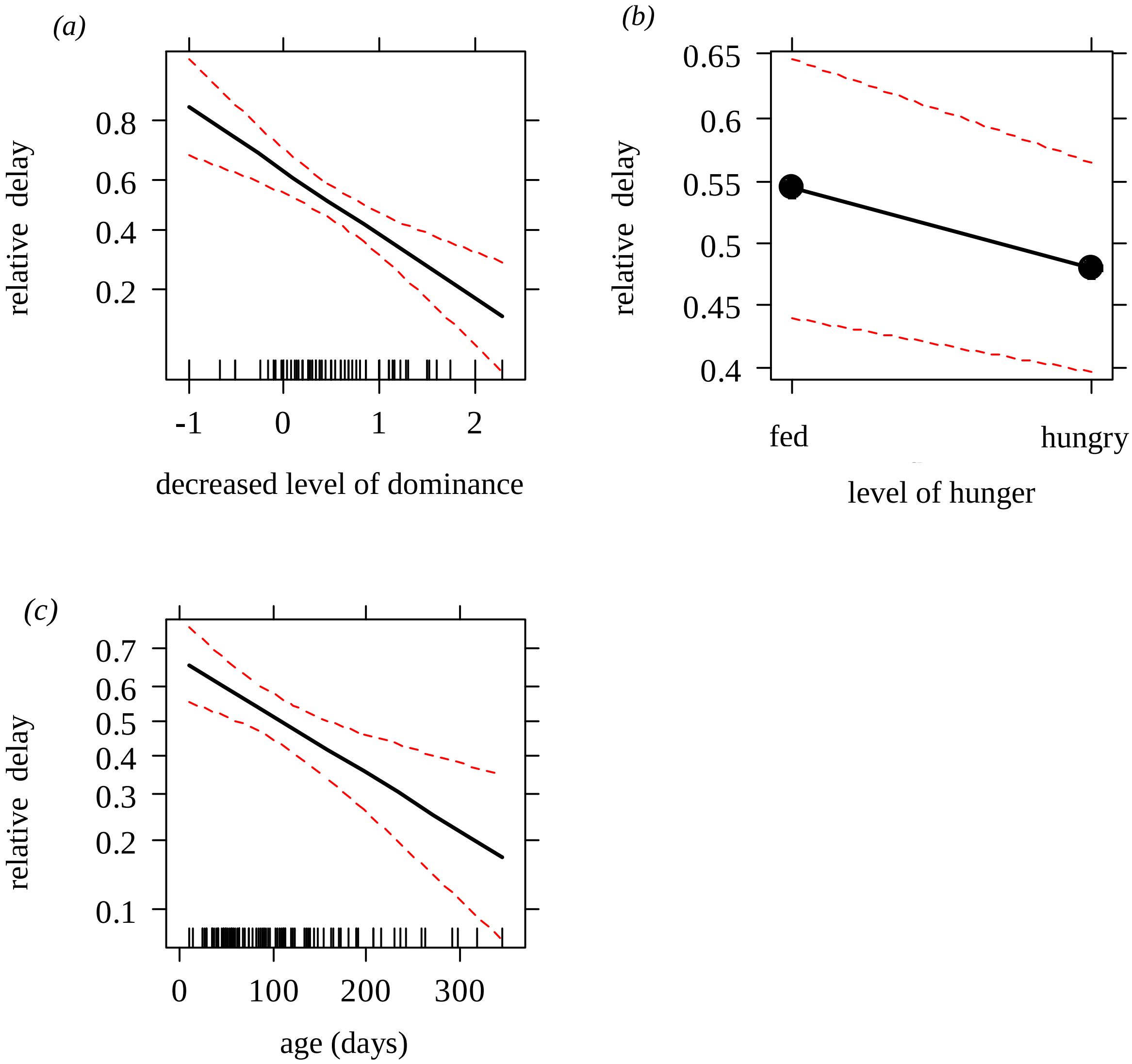
We modelled (1) the relative delay (*rel. delay*) as a quasibinomial distribution (logit link), since dominant and subordinate mean latencies as input parameters for *rel. delay* were not binomial data such as "success" or "failure" but had been used to create a standardised proportion. We rounded the time lost by the dominant (2) *domlat* and by the subordinate (3) *sublat* to obtain counts of seconds when they were used as response variables and modelled them as poisson distributions (log link). We excluded from this analysis the cases (5 from 97 focal samples) where the subordinate did not return to suckling after an agonistic interaction (*sublat* = minimum of 900 seconds).

Results

Relative delay (skew by dominants)

The relative delay between both sibling latencies declined when: (1) the level of dominance exerted by the dominant diminished (supplementary figure 4a); (2) when litters had not recently been fed (supplementary figure 4b); (3) as litter age increased within the first 120 days (supplementary figure 4c, “model 1”, supplementary table 4).

Supplementary figure 4 The relative delay of the dominant sibling in terms of access in comparison to the subordinate following aggression by the dominant, in relation to (a) the decreasing level of dominance (*mean.score*), (b) the level of hunger of the litter and (c) litter age in days, as predicted by model 1 in ESM3. The tick marks inside the x-axis indicate the data points and the dotted line indicates the confidence interval. The unequal spacing on the y-axis is used to obtain straight lines (see ESM 2 for further information on the methods used to produce figures 1 to 3). The relative advantage of the dominant is higher when it tends towards 1, i.e. when the dominant returned to suckle faster (had a shorter latency) than the subordinate.

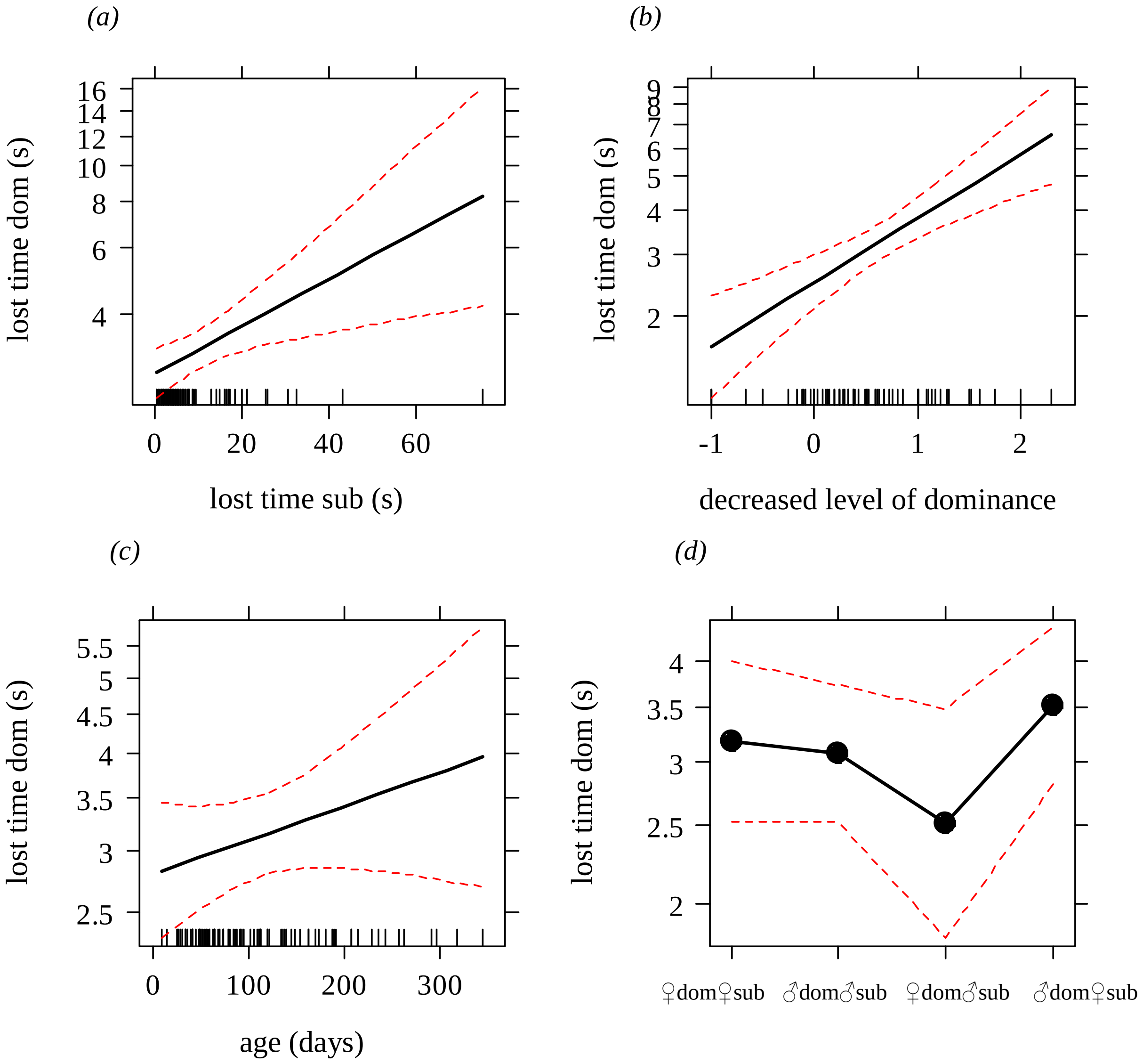


Factors influencing the time lost suckling by the dominant

The suckling time lost by dominant siblings owing to their own aggression increased (1) as subordinates increased their latency to return to suckling (supplementary figure 5a); (2) as the level of dominance decreased (supplementary figure 5b); and as (3) litter age increased within the first 180 days (supplementary figure 5c). Suckling time lost by dominants was lowest when the dominant was female and the subordinate was male and it was highest when the dominant was male and the subordinate was female (supplementary figure 5d).

Supplementary figure 5 : The mean lost suckling time (s) of the dominant sibling owing to its aggression (*domlat*) in relation to (a) the time (s) lost suckling by the subordinate (*sublat*), (b) the decreasing level of dominance of the dominant (*mean.score*), (c) litter age in days and (d) the sex composition of the litter (*scomp2*) where ♀dom♀sub= all-female litter;

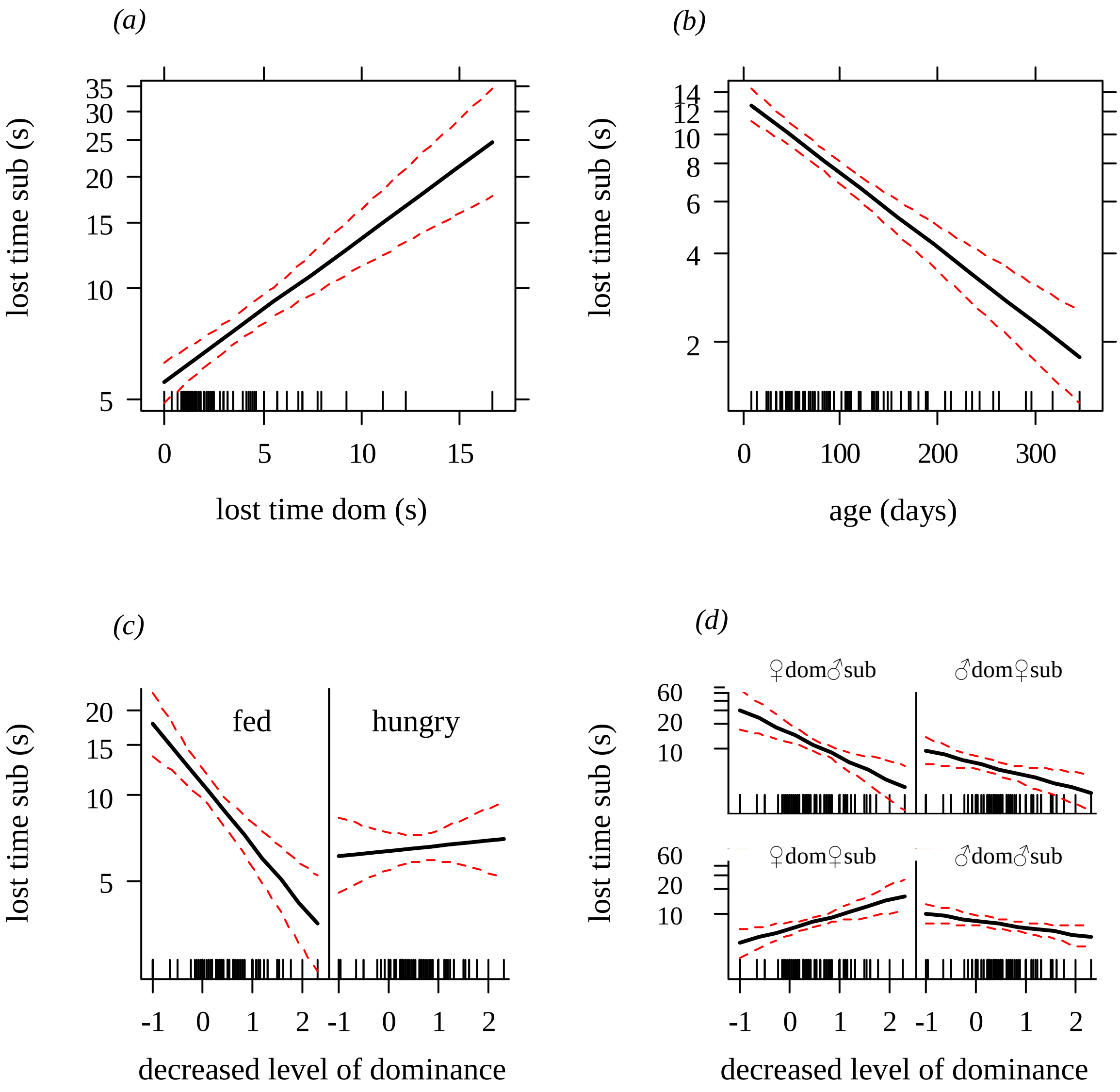
♂dom♂sub= all-male litter; ♀dom♂sub= mixed-sex litter with dominant female; ♂dom♀sub= mixed-sex litter with dominant male, as predicted by model 2. The tick marks on the x-axis indicate the data points and the dotted line shows the confidence interval.



Factors influencing the time lost suckling by the subordinate

The suckling time lost by subordinate siblings following aggressive actions by dominants (1) increased as the mean suckling time lost by dominants increased (supplementary figure 6a), (2) decreased with litter age within the first 100 days (supplementary figure 6b) and (3) decreased with the decreasing level of dominance in recently fed litters (supplementary figure 6c). The suckling time lost by subordinate siblings declined with the decreasing level of dominance in mixed-sex litters when the dominant was female and the subordinate was male, and it increased in all-female litters (“model 3”, supplementary table 4, supplementary figure 6d).

Supplementary figure 6 : The mean lost suckling time (s) of the subordinate owing to aggression by the dominant (*sublat*) in relation to (a) the time (s) lost suckling by the dominant, (b) litter age in days, (c), the interaction effect between the decreasing level of dominance (*mean. score*) and the level of hunger of the litter and (d) the interaction effect between the decreasing level of dominance and the sex composition of the litter (*scomp2*) where ♀dom♀sub= all-female litter; ♂dom♂sub= all-male litter; ♀dom♂sub= mixed-sex litter with dominant female; ♂dom♀sub= mixed-sex litter with dominant male, as predicted by model 3. The tick marks on the x-axis indicate the data points and the dotted line shows the confidence interval.



Supplementary table 4 Summary of the best models with smallest Akaike Information Criterion (AIC) fitted to predict variation in (1) the relative delay between siblings (*rel. delay*), (2) the suckling time lost by the dominant (*domlat*) owing to its own aggression and (3) the suckling time lost by the subordinate sib owing to the aggression of the dominant sib (*sublat*). ΔAIC is the difference between the AIC of the intercept model and the AIC of the candidate model. All models included litter identity as a random effect.

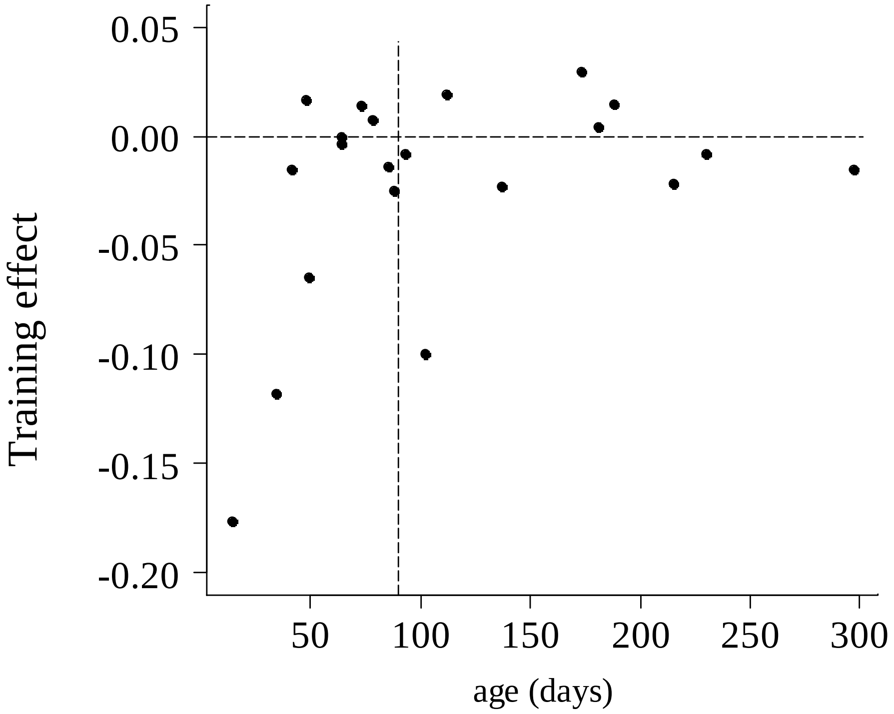
selected	best models (with similar AIC (2 units))	AIC	ΔAIC
	relative delay between siblings (*rel. delay*)		
	mean. score, age120†	48.52	8.65
✓(model 1)	*mean. score, age120*†, *hunger*	50.29	10.10
	time lost suckling by the dominant (*domlat*)		
	sublat9, mean.score, age180	110.66	36.25
✓(model 2)	*sublat9, mean.score,age180,scomp2*	112.00	34.90
	time lost suckling by the subordinate (*sublat*)		
✓(model 3)	*domlat, age100, hunger*mean.score*‡, *scomp2*mean.score*‡	245.53	105.154

For abbreviation see article and supplementary table 2 in ESM1.

Training effects

The intensity of the submissive response of a subordinate in response to the aggression by the dominant during the course of a focal sample is here measured by the variable *score*. *score* declined strongly during focal samples in the youngest litters, resulting in a negative slope of the regression of *score* on focal sample time. As the age of a litter increased, the regression slopes of *score* against time within a focal sample tended to increase (log transformed linear regression of age, $F_{1,18} = 7.722$, $P = 0.012$, adjusted $r^2 = 26.13$, supplementary figure 7).

Supplementary figure 7 Change in the level of dominance during repeated aggression interactions during a focal sample in relation to the age of the litter (in days). The change in behaviours was expressed as the slope of regressions, i.e. the change of *score* over consecutive interactions in time. Highly negative values indicate a strong loser effect; values close to zero indicate little change in response during a focal sample. The three most negative values correspond to three different litters. The vertical dotted line illustrates the change in tendencies from 90 days of age, approximately the mean age before which siblicide occurs [4,25].


Training effect
0.05
0.00
-0.05
-0.10
-0.15
-0.20
50
100
150
200
250
300
age (days)

CHAPTER 3

Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in spotted hyenas (*Crocuta crocuta*)

(minor revisions)

SARAH BENHAIEM, MARTIN DEHNHARD, ROBERTO BONANNI, HERIBERT HOFER,
WOLFGANG GOYMANN , KLAUS EULENBERGER, MARION L. EAST

Author contribution:

The framework of the study, in terms of the development of an appropriate assay was developed by Martin Dehnhard (MD), Marion L East (ME), Roberto Bonanni (RB) and Wolfgang Goymann (WG). The concept of the study on juvenile animals was provided by ME, Sarah Benhaiem (SB) and Heribert Hofer (HH). Faecal sample collection for the radiometabolism study in Leipzig Zoo and in Amerstfoort Zoo was organised by ME. Anaesthesia and administration of the radio-labelled hormone were done by Klaus Eulenberger (KE). Faecal extracts (ACTH challenges) were prepared and provided by WG. Faecal samples of Serengeti free-ranging spotted hyenas were collected by SB and ME. Faecal extraction was done by SB. The cortisol-3-CMO was developed by MD. The measurement of hormone concentrations with ELISA was organised by MD. Statistical analysis was done by SB. The manuscript was primarily written by SB, ME and MD. RB, WG, HH and KE commented on the manuscript.

Running title: Corticosteroids in spotted hyenas

Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in spotted hyenas (*Crocuta crocuta*)

SARAH BENHAIEM[1], MARTIN DEHNHARD[1], ROBERTO BONANNI[1,2], HERIBERT HOFER[1], WOLFGANG GOYMANN[3], KLAUS EULENBERGER[4], MARION L. EAST[1]

[1] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

dehnhard@izw-berlin.de; direktor@izw-berlin.de; east@izw-berlin.de

[2] Dipartimento di Biologia Evolutiva e Funzionale, Università degli Studi di Parma, via G.P. Usberti 11/A, 43100 Parma, Italy

rbonanni75@gmail.com

[3] Max-Planck-Institut für Ornithologie, D-82319 Seewiesen, Germany

goymann@orn.mpg.de

[4] Zoo Leipzig GmbH, Pfaffendorfer Str. 29, 04105 Leipzig, Germany

keulenberger@zoo-leipzig.de

Corresponding author: benhaiem@izw-berlin.de,

phone: +49- 30-51-68-520, fax: +49-30-516-81-10

http://dx.doi.org/10.1016/j.ygcen.2012.05.006

Abstract: The use of enzyme immunoassays (EIAs) to measure faecal glucocorticoid metabolites (fGCM) is a useful technique for the non-invasive monitoring of adrenocortical activity in vertebrates. Each EIA should be validated for each species to which it is applied. The objective of this study was to validate an EIA (cortisol-3-CMO) for the measurement of fGCM concentrations in spotted hyenas. We demonstrate that this assay detected a significant increase in fGCMs after an adrenocorticotropic (ACTH) hormone challenge in two captive animals and a significant increase in fGCMs in another captive animal after anaesthesia. High-performance liquid chromatography (HPLC) immunograms were used to characterise fGCM in samples from a fourth captive hyena that received an i.v. injection of [^{3}H] cortisol. All HPLC fractions were analysed with the EIA for the presence and quantities of radiolabelled fGCM. Radiolabelled fGCM consisted of substances with a higher polarity than cortisol and of less polar substances that eluted between cortisol and corticosterone. Authentic radiolabelled cortisol was not detected. The cortisol-3-CMO assay measured substantial amounts of immunoreactivity corresponding to the radioactive peaks. We conducted pairwise comparisons of fGCM concentrations in individual free-ranging juvenile spotted hyenas when less than 6 months of age and when between 6 and 24 months of age. We expected juveniles to experience a more unpredictable and therefore more stressful environment when younger than when older. When younger, juveniles had indeed significantly higher fGCM concentrations than when they were older. Our results demonstrate that our assay can be used to assess adrenocortical activity in spotted hyenas.

Key-words: spotted hyena, faecal glucocorticoid metabolites, enzyme immunoassay, radiolabelled metabolites, ACTH, age effect

1. Introduction

Stressful stimuli (stressors) activate the hypothalamic–pituitary–adrenal (HPA) axis, causing the release of glucocorticoids, i.e., steroid hormones such as cortisol or corticosterone, which induce behavioural and physiological changes [19,32,40,48]. Stressors can cause a rapid adaptive short-term (‘acute’) release of glucocorticoids, leading to the mobilisation of energy and behavioural changes, or a long-term (‘chronic’) response that may decrease fitness by impairing growth [57] or cognition [38], by inducing immunosuppression and atrophy of tissues [40], and by suppressing reproductive performance [12,36]. Thus, the measurement of glucocorticoid concentrations can provide useful information on the health of captive and free-ranging wildlife populations [26,29,46,58].

Glucocorticoids are secreted in pulses into the blood; their secretion may fluctuate on a circadian basis [39]. Therefore blood samples contain plasma glucocorticoid levels that are only representative for a narrow time frame. Baseline levels of glucocorticoids are defined as levels of glucocorticoids in the absence of acute stressors [56]. Capture or anaesthesia of animals to obtain blood samples are often perceived as stressors by animals as both lead to a rapid and significant increase in circulating glucocorticoid levels [16,48,61]. Thus, blood samples are usually of little value to characterise baseline glucocorticoid secretion unless they were obtained by very rapid sampling [47] or the application of ingenious methods for blood sampling that do not involve handling (e.g. [2,55]).

By contrast, non-invasive methods to assess faecal glucocorticoid metabolite (fGCM) concentrations do not alter the physiological parameter under investigation and may therefore be more appropriate for the measurement of factors influencing adrenocortical activity, especially in free-ranging animals. These methods have been established for a variety of vertebrate species (see [53]) using hormone assays such as enzyme immunoassays (EIA) (reviewed most recently by [29]). In contrast to blood sampling procedures, they allow the collection of repeated samples from the same individual without influencing fGCM concentrations [6,53]. fGCM levels represent an integrated average measure and are therefore less affected by transient increases of glucocorticoids into the bloodstream than blood samples [30].

Even so, determination of fGCM may be challenging. First, authentic cortisol or corticosterone are very rarely found in the faeces of animals [42] and assays that are used for non-invasive measurements rely on the ability of the antibody to detect the relevant metabolic breakdown products [18,41]. Second, researchers need to demonstrate that the concentrations of fGCM change in step with stressors experienced by the animal. Therefore, assays used to measure fGCM should be biologically validated for each species [53] before they are used to measure fGCM in a particular species.

The aim of this study was to validate an EIA to measure fGCM in a large social carnivore, the spotted hyena (*Crocuta crocuta*), by focusing on captive adults and free-ranging juveniles. The spotted hyena (*Crocuta crocuta*) is a social carnivore that lives in large, fission-fusion groups called clans [34]. The social environment of juveniles spotted hyenas is structured by the adult female dominance hierarchy and the fact that all females and their offspring are socially dominant over all immigrant males [11,34,51]. The social position of juvenile spotted hyenas in their natal clan is determined by the support provided by their mother during interactions with other clan members and as a result the position held by a spotted hyena when it attains adulthood is close to and below that held by its mother [11,51]. During a period that probably spans many months after birth, juveniles learn to identify the numerous members of their clan and to correctly assess their relative position to all these animals. Juveniles are unlikely to be proficient at identifying all members of their clan during their initial period of social learning and also may not be able to accurately predict whether to expect an aggressive response during agonistic interactions with clan members, particularly when their mother is absent. For these reasons, the social environment of young juveniles is likely to be less predictable and thus more stressful than that of older juveniles.

The movements of migratory herbivores in the Serengeti National Park, northern Tanzania, have a profound effect on the ability of lactating spotted hyenas to attend their dependent cubs at the clan communal den [17,25,28]. When their mothers are absent on long-distance foraging trips to distant concentrations of migratory herbivores, young juveniles that are left at the communal den face an unpredictable period that may last for several days, during which they are not nursed [24] and thus experience hunger, weight loss and dehydration [25]. When spotted hyenas are young and completely dependent on maternal milk, they are more likely to experience these potentially stressful effects than when they are older.

Animals that cannot predict the onset of stressful stimuli release more glucocorticoids that animals that can; thus the control (or lack of control) over stressors is an important determinant of the intensity of the stress response [3,4,60]. For the reasons mentioned above, young juvenile spotted hyenas are likely to have less control over stressful stimuli and thus are expected to have higher fGCM concentrations than when they are older juveniles. To our knowledge, such an age effect on GC concentrations in spotted hyenas has not been investigated.

Previously, an EIA based on a corticosterone antibody purchased from ICN Biomedicals was validated [18] and used to compare faecal corticosterone metabolite concentrations among adult female spotted hyenas [19]. Later, commercial corticosterone radioimmunoassay kits based on the same antibody (MP Biomedicals, formerly ICN) were used to investigate which social, ecological and anthropogenic factors influence faecal corticosterone metabolite concentrations in adult spotted hyenas [54]. In this study, the objectives were to: (1) validate whether the cortisol-3-CMO assay can be used to quantify fGCM in the spotted hyena. For this we conducted an ACTH challenge experiment and an anaesthesia to induce stress, and applied a radiometabolism study to characterise the fGCM of spotted hyena which react with the cortisol-3-CMO assay, (2) provide the first data on the concentration of fGCM in juvenile spotted hyenas to investigate whether younger juveniles have higher fGCM concentrations than when they are older.

2. Methods

2.1. Captive animals and sample collection

All samples from captive animals were collected by animal keepers within the animal management schedule of each zoo. This resulted in variation (ranging between 0 and 12h) in the time lag between defecation and sample collection. Typically, faeces produced during the night were collected and frozen early the following morning. The length of time elapsed between faeces collection and freezing (up to 12 hours) did not affect fGCM levels in a population of free-ranging adult spotted hyenas (N= 65 individuals, [54]) and is therefore unlikely to have affected the values of fGCM measured in this study.

2.1.1. Radiometabolism study

To characterise fGCM contents, we performed a radiometabolism study on one adult male spotted hyena at Leipzig Zoo, Germany in 2008. 2.25 ml of a sterile 0.9% NaCl solution was added to a solution (0.25ml) containing ~250 µCi 1,2,6,7-[^{3}H] cortisol (TRK407, Amersham Bioscience, UK) in ethanol. The animal was anesthetised and the total volume was injected into the cephalic vein. We collected two faecal samples in the 24 hours preceding the injection and three faecal samples during the 72 hours after the radiolabelled injection. Aliquots of each sample were extracted for fGCM determination and radioactivity counting. Samples were frozen at - 20°C until analysed. For the HPLC analyses we used the sample containing the highest amount of radioactivity, which was the sample collected 47 h after injection.

2.1.2. ACTH challenge

The administration of adrenocorticotropic hormone (ACTH) stimulates the secretion of glucocorticoids; glucocorticoid metabolites can then be measured in faeces several hours later (depending on gut passage time, see [42]). To test the efficiency of the cortisol-3-CMO assay to detect adrenocortical activity, we used faecal methanol extracts (see section *2.3.* for details) obtained for an ACTH challenge experiment that was conducted in 1998 in Dvur Králové Zoo (Czech Republic) [18] on two captive adult spotted hyenas (one male and one female). Both animals were dart-injected with 200 IU of an ACTH preparation. We used a total of 39 faecal methanol extracts (17 extracts from the male and 22 extracts from the female). These extracts were stored frozen (at -80°C for the initial 6 years and at -20°C for the following 6 years) until analysed. For details on the experiment see [18].

2.1.3. Anaesthesia as a stressor

Faecal samples were collected from one adult male at Amersfoort Zoo (Netherlands) in 2011 which was anaesthetised for a veterinary health investigation. Faecal samples were obtained for 2 days preceding anaesthesia and for 4 days after that and were stored at -20°C until analysis.

2.2. Free-ranging animals and sample collection

We collected 280 faeces from 107 individually known juvenile spotted hyenas (44 females and 63 males) aged between 84 and 707 days between June 2007 and December 2009. Study animals belonged to three large clans in the Serengeti National Park in Tanzania, East Africa (for details of study area and study clans see [5,10]. Our study clans are relatively large and contained approximately 85 animals each during this study. In the Serengeti National Park, juvenile spotted hyenas less than six months of age are entirely dependent on maternal milk and are stationed at the clan communal den [24,25,27,28]. Animals were considered adults at 24 months [11]. Therefore we placed juvenile spotted hyenas below six months of age in our younger juvenile category and juveniles between six and 24 months of age in the older juvenile category. We recognised juvenile hyenas by their distinctive spot patterns [14,22] and ear notches, scars and bald patches [17]. The age of juveniles was estimated in days using pelage characteristics, whether their ears were flattened or upright, and their balance and coordination during locomotion [8,43]. Estimates of age were accurate to within 7 days. To determine sex, we used the dimorphic glans morphology of the erect phallus in either sex [15]. Individuals were sexed at the age of approximately 3 months.

Faeces were immediately collected following a defecation. To conform to the recommendations by [31], faeces were first stored in the field in a cold box for less than 3 hours after collection. Prior to storage, faeces were mechanically mixed and subsamples of faeces stored in 5 ml tubes in either liquid nitrogen or frozen at approximately -10 °C in the Serengeti until transported frozen to Germany where they were stored at - 80°C until analysed.

2.3. Processing of faecal samples

Faecal samples were thawed for approximately one hour. For the extraction of fGCM from the faecal matrix, we suspended (and shook for 30 minutes) 0.5 g of wet faecal samples in 4.5ml of 90 % methanol. After centrifugation (1200 x *g*, 15min) the supernatant was transferred into a new tube and diluted 1:1 with water. Aliquots of the faecal extracts were subjected either to HPLC analysis or directly to the cortisol-3-CMO assay.

2.4. High performance liquid chromatography (HPLC)

A Dionex system equipped with a quaternary pump (P580), an ASI autosampler, and a column oven (STH 585) were used for chromatographic separation (Dionex GmbH, Idstein, Germany). For separation and characterisation of cortisol metabolites, 100 µl of faecal extracts were loaded on a reverse-phase Ultrasep ES100/RP—18/6 µm HPLC column (4 x 250mm, Sepserv, Berlin, Germany). Metabolites were separated with a mixture of methanol and water (75 + 25) at a flow rate of 1 ml/min. Fractions of 0.33ml were collected at 20 s intervals over a period of 21 min using a FRAC-200 fraction collector (Pharmacia Biotech, Freiburg, Germany) and diluted with one volume of water. To generate the HPLC immunograms, 20 µl of the fractions were subjected to the cortisol-3-CMO assay. The elution positions of authentic cortisol, corticosterone, testosterone and dihydrotestosterone (Sigma Chemie GmbH, Deisenhofen, Germany) on this column were previously determined by separate HPLC runs. Both androgen standards were analysed with a testosterone microtitre plate enzyme immunoassay as described by [33].

2.5. Enzyme Immunoassay

Faecal cortisol metabolite analyses were carried out with an in-house microtitre plate enzyme immunoassay using a polyclonal antibody raised in rabbits against cortisol-3-CMO-BSA and cortisol-3-CMO-peroxidase as label. The antibody cross-reactivities to different steroids were as follows: 4-pregnen-11α,17,21-triol-3,20-dione (cortisol), 100%; 4-pregnen-21-ol-3,20-dione (desoxycorticosterone), 6.3%; and <0.1% for 4-pregnen-3,20-dione (progesterone), 5α-pregnane-3,20-dione, 5α-pregnane-3β-ol-20-one, dexamethasone, estradiol, and testosterone.

To test for parallelism we used serial dilutions of faecal extracts from two individuals and compared displacement curves of both samples with the displacement curve produced by the steroid standard. After a log transformation (to base 10) of the concentration to obtain a linear relationship, we used an analysis of covariance to test whether the slopes of the displacement curves generated from serial dilutions of two faecal extracts from two individuals and the standard curve differed from each other. The slopes of the displacement curves did not differ from each other, thereby demonstrating parallelism (p-value of the interaction: $P = 0.93$)

We performed measures in duplicate and accepted results only if the coefficient of variation was ≤ 5%. Intra- and inter-assay coefficients of variation were 16.5 % and 23.1% for a low (N=9) and 5.3% and 10.1% for a high concentration pool (N=11). Mean sensitivity of the assay at 90% binding was 0.5 pg per well. Final concentrations of fGCM were expressed as ng/g faecal matter.

For analyses, microtitre plates were coated with goat anti-rabbit IgG (4°C, overnight), blocked with BSA in assay buffer and after 30 min at 22°C, the excess of blocking buffer was decanted and plates were stored at -22°C. Prior to use, plates were washed once with washing solution (0.05% Tween 80) using an automated microtitre plate washer (SLT 96PW; Tecan, Crailsheim, Germany). Duplicates of 20 µl faecal extract or cortisol standards prepared in 40% methanol ranging from 0.2 to 100 pg/20 µl were simultaneously pipetted into respective wells along with 100 µl Cortisol-HRP conjugate in assay buffer (50 mM Na_2HPO_4/Na_2HPO_4, 0.15 M NaCl, 0.1% BSA, pH 7.4) with the aid of diluter dispenser.

Then, 100 µl cortisol-specific antibody was added. After overnight incubation at 4°C, plates were washed four times and 250 µl tetramethylbenzidine/H_2O_2 substrate solution was added. After incubation (45 min, 4°), the reaction was stopped with 50 µl H_2SO_4 and absorbance was measured at 450 nm with a 12-channel microtitre plate reader (Infinite M 200; Tecan). Hormone concentrations were calculated using Magellan software (Tecan).

Similar cortisol-3-CMO assays have been validated for the measurement of fGCM in several primate species such as Barbary macaques *(Macaca sylvanus)*, lowland gorillas *(Gorilla gorilla)* and common marmosets *(Callithrix jacchus)* [21] and in domestic dogs (*Canis lupus familiaris*) [50].

2.6. Statistics

For all statistical procedures we used the R.2.11.1 (R Development Core Team 2010) software. The threshold for significance was fixed at 5% and all tests were two-tailed. Statistics are listed as means ± SD. Increases in fGCM excretion after ACTH challenge and anaesthesia were considered significant if they exceeded mean baseline concentration plus three standard deviations. To test whether fGCM levels of juvenile spotted hyenas were higher in the youngest age category we used one sample per individual. When several samples were collected from one individual within one age category, we used the mean value of fGCM concentrations per individual per age category to avoid pseudo-replication. Because differences in glucocorticoid metabolism may exist between the sexes (as in rodents: [35], [52]; and birds: [45]) we first compared fGCM levels between males and females in each age category using a Mann-Whitney U test. We then compared within-individual changes in fGCM level in the two age categories using a Wilcoxon signed-rank test.

3. Results

3.1. HPLC analysis

HPLC profiles of radiolabelled cortisol metabolites in the faeces of the Leipzig Zoo spotted hyena and the elution positions of immunoreactivity determined by the cortisol-3-CMO assay are shown in figure 1. The faecal extract of the Leipzig Zoo hyena was composed of several radiolabelled metabolites that were detected in fractions 5-6, 10-13, 16-17, and 31–32, with the majority in fractions 5-6. Because their polarity exceeded that of cortisol we suggest the components in fractions 5-6 are conjugated metabolites. A large amount of polar metabolites eluted between cortisol and corticosterone (fractions 10-13), followed by less polar substances at fractions 16-17. The last, unpolar metabolites (fractions 31-32) eluted between the standards of testosterone (T; 4-androsten-17β-ol-3-one) and dihydrotestosterone (DHT; 5α-androstan-17β-ol-3-one) and probably represented androgens resulting from cortisol metabolism. Our assay detected cortisol immunoreactivity consisting of five major peaks (fractions 5-6, 9-10, 11-12, 26–28 and 32), of which the first three clearly and the last one corresponded to radiolabelled metabolite peaks. The immunoassay also detected a minor radiolabelled metabolite peak in fractions 16-17. The minor peak of immunoreactive metabolites detected in fractions 26-28 did not correspond to any of the radiolabelled metabolites.

3.2. ACTH challenge

Administration of ACTH resulted in a significant increase of fGCM concentrations in both study individuals. The ACTH-induced peak in fGCM occurred 16 ± 6 h (h ± range) (in the male, figure 2a, 17 samples) and 32 ± 1 h (in the female, figure 2b, 22 samples) after injection. fGCM concentrations returned to pre-treatment levels within 101 ± 1 h (male) and 58 ±

1 h (female), respectively. With our assay we measured significant increases of fGCM from pre-treatment concentrations of 50.2 ± 20.4 ng/g (12 samples) to 576.0 ng/g in the faecal extracts of the male and from 37.3 ± 13.7 ng/g (12 samples) to 913.9 ng/g in the faecal extracts of the female. This is equivalent to a 12-fold and 21-fold increase over the pre-treatment levels, respectively. The range of values measured by our EIA was similar to that found by [18] using an ICN-corticosterone assay (male: range 27.6 – 576.0 ng/g with our assay vs. 14.9 - 321.7 ng/g with the ICN-corticosterone assay; female: range 13.3 – 913.9 ng/g with our assay vs. 11.7 - 783.7 ng/g with the ICN-corticosterone assay).

3.3. Anaesthesia

Anaesthesia resulted in an increase in fGCM concentrations in the male hyena at Amersfoort Zoo (figure 3). fGCM increased from pre-anaesthesia concentrations of 2.1 ± 0.4 ng/g (3 samples) to 5.3 ng/g on day 2 (two days after anaesthesia), and reached a maximum concentration of 12.5 ng/g on day 4. The maximum level of fGCM on day 4 was significantly higher than the initial level.

3.4. Faecal glucocorticoid metabolite concentrations in juvenile hyenas

There were no sex differences in fGCM concentrations in the younger juveniles (males: 45.24 ± 69.60 vs. females: 35.39 ± 37.99, Mann-Whitney U test, U = 884.5, *P* = 0.731, N = 50 males and N = 37 females) or in the older juveniles (males: 17.70 ± 11.31 vs. females: 19.24 ± 13.79, U = 416, *P* = 0.791, N = 31 males and N = 28 females). Hence, male and female data were considered together in the following analysis.

Consistent with our prediction, concentrations of fGCM metabolites were significantly (Wilcoxon signed rank test: V = 646, N = 39, *P* = 0.0002, figure 4) higher when juveniles were young (51.0 ± 72.6 ng/g; range 8.9 to 377.4 ng/g, mean age 131.2 ± 29.8 days) than when they were older (18.9 ± 14.2 ng/g; range 4.6 to 76.6 ng/g, mean age 304.7 ± 115.3 days).

4. Discussion

The EIA (cortisol-3-CMO) that we tested measured significant increases in fGCM concentrations in spotted hyenas (1) after ACTH challenge experiments on two captive animals and (2) after the anaesthesia of one captive animal. The radiometabolism study confirmed that the EIA detected most of the glucocorticoid metabolites present in the faeces of spotted hyenas. These results provide strong evidence that the cortisol-3-CMO EIA can be used to assess adrenocortical activity in this species. Furthermore, in accordance with our prediction we found significantly higher fGCM concentrations in free-ranging young juvenile spotted hyenas than when they were older juveniles.

The cortisol-3-CMO EIA measured 12-fold and 21-fold increases in fGCM concentrations after ACTH challenge in two animals (figure 2). The observed concentrations were similar in range (peak and pre-treatment values) to those found by a previous study that used a different antibody to measure fGCM concentrations in the same faecal methanol extracts assessed by our study [18]. Our results also demonstrate that faecal steroids stored as faecal methanol extracts were stable for many years when frozen and provide good evidence that the assay applied in the present study is suitable to assess adrenocortical activity in spotted hyenas.

The HPLC immunogram showed both the affinity of the antibody in the cortisol-3-CMO assay for different cortisol metabolites produced by spotted hyenas and the amount of these metabolites in a particular HPLC fraction. Although HPLC profiles for radiolabelled and immunoreactive metabolites were not identical, the overlap in peaks between both profiles confirmed that our EIA detected the metabolites derived from cortisol metabolism. As expected, authentic cortisol was mostly absent from spotted hyena faeces, similar to yellow baboons (*Papio cynocephalus*) [59] and several other carnivores [63]. The HPLC profile of radiolabelled cortisol metabolites suggested that the cortisol-3-CMO assay mainly detected excreted cortisol metabolites rather than pure cortisol.

Only a single peak containing a minor amount of immunoreactive metabolites (figure 1, fractions 26-28) was not supported by adequate radiolabelled substances. This peak may represent metabolites derived from gonadal rather than adrenocortical activity. Although such metabolites have been detected in the faeces of other species (e.g. [59]), little attention has been paid to their potential influence on fGCM measurements. Alternatively, these substances may be the consequence of side-chain cleavage at C-17 in extensively metabolised glucocorticoids which lead to the formation of androstanes bearing an oxygen atom at position C-11 and which are similar to androgen metabolites [42]. Assays that measure faecal testosterone metabolites and those designed to measure faecal cortisol metabolites cannot necessarily differentiate between substances derived from gonadal or adrenocortical activity. Nevertheless, radiometabolism experiments provide a useful method to assess the extent to which an EIA antibody cross-reacts with metabolites derived from steroid hormones other than the radiolabelled target hormone. They therefore constitute a necessary step for the validation of an EIA for fGCM measurements in a species.

Conventional methods to measure glucocorticoids in animals have relied upon analysis of serially collected blood samples [40,46]. In domestic or wildlife species, whether in captivity or free-ranging, regular blood sampling is undesirable since it requires handling, restraint and anaesthesia of animals. Our results confirm that handling methods such as anaesthesia stimulate the production of glucocorticoids (figure 3, see also [13,16,63]). Anaesthesia of one captive animal resulted in a 6-fold increase in fGCM concentrations on the fourth day after anaesthesia, roughly 96 hours after treatment.

The fGCM concentrations measured by this study in one captive individual before anaesthesia (ca. 2 ng/g, figure 3) and in older juvenile free-ranging spotted hyenas (figure 4), were similar to those (ca. 5 ng/g) reported by [18] for one captive spotted hyena before it was translocated. The fGCM values we measured in two other captive hyenas before an ACTH experiment in another zoo were higher (50 ng/g in the male and 37 ng/g in the female, figure 2), but considerably less than those reported in captive spotted hyenas before an ACTH experiment that were measured with a radioimmunoassay kit (from 225 to 325 ng/g, six hyenas, [7]). As fGCM levels may also substantially vary between samples collected on consecutive days [62], these values illustrate the necessity of establishing pre-treatment fGCM levels based on multiple samples, prior to any experimental procedure.

In accordance with our prediction that young juvenile spotted hyenas in the Serengeti National Park experience a less predictable and therefore more stressful environment than when they are older juveniles, our results revealed higher fGCM concentrations in individuals when they were young. These results probably represent the combined negative effects of several stimuli that have their greatest impact within the first six months of life.

In the normally stable social structure of a spotted hyena clan, social instability can result in significantly elevated levels of fGCM [20]. This suggests that an unpredictable outcome to a social interaction is a stress-inducing stimulus in spotted hyenas. It is plausible that the social environment experienced by a juvenile spotted hyena during its first six months of life is less predictable and thus more stress-inducing than when it is older, has gained in social competence and is better established in the clan social hierarchy. When stationed at the clan’s communal den, an important centre of group social activity [5,9], the rate at which younger juveniles experienced unpredictable or aggressive responses from other clan members may be higher than when they are older and range more widely. The fission-fusion structure of clans probably allows older juveniles not permanently based at the communal den to reduce their rate of encounters with clan members that they have learnt to avoid.

Juvenile spotted hyenas left unattended for periods of days when their mothers are on long-distance foraging trips [23,24] also experience unpredictable periods of hunger, dehydration and weight loss [25,28]. These are likely to induce a greater stress response in younger juveniles entirely dependent on maternal milk (when less than six months) than older, less dependent juveniles. Furthermore, when mothers are absent, their offspring lack maternal support during interactions with other clan members. This may be more stressful to younger than older juveniles since clan members are more likely to respond aggressively for younger than older juveniles when their mother is absent than when she is present [51].

A higher sensitivity to stressors during the first months of life has been reported in several mammalian species. For instance, juvenile common marmoset (*Callithrix jacchus*) [44] and chimpanzee (*Pan troglodytes*) [1] have higher basal cortisol values than older ones. Juvenile Belding's ground squirrels (*Spermophilus beldingi*) have higher levels of cortisol during the first 5 days after emergence than during subsequent days. At this early age they are in the process of becoming nutritionally independent and are very vulnerable to predation and infanticide by adult conspecifics [37]. Their increased levels of glucocorticoids may facilitate rapid learning and acquisition of anti-predator behaviours [38]. As spotted hyenas show complex social behaviours and a structured linear hierarchy, elevated levels of glucocorticoids during the critical first six months of life might play an important role in their learning of social conventions during interactions with other clan members.

In conclusion, we have validated a new non-invasive method for monitoring adrenocortical activity in the spotted hyena. In contrast to all previous studies of fGCM in this species which focused on adults, our study is the first to investigate fGCM levels in juvenile free-ranging spotted hyenas.

ACKNOWLEDGEMENTS

We are grateful to the Amersfoort Zoo, particularly T. Hebdrikse and R. van der Meer and the Leipzig Zoo, particularly Dr G. Nötzold, Dr S. Langguth and T. Lässig. We thank M. Rohleder and K. Paschmionka for their technical support and B. Wachter, E. Davidian and O. Höner for helpful insights. We are grateful to the Tanzania Commission for Science and Technology for permission to conduct the study, and the Tanzania Wildlife Research Institute and Tanzania National Parks for their support of the research. We thank A. Francis, T. Shabani in Tanzania and D. Thierer and K. Wilhelm in Berlin for their assistance. Finally, we thank the German Academic Exchange Service (DAAD) and the Leibniz Institute for Zoo and Wildlife research for funding.

REFERENCES

[1] S.F. Anestis, Behavioral style, dominance rank, and urinary cortisol in young chimpanzees (*Pan troglodytes*), Behaviour 142 (2005) 1245-1268.

[2] J.M. Arnold, S.A. Oswald, C.C. Voigt, R. Palme, A. Braasch, C. Bauch, P.H. Becker, Taking the stress out of blood collection: comparison of field blood-sampling techniques for analysis of baseline corticosterone, J. Avian Biol. 39 (2008) 588-592.

[3] J.R. Averill, Personal control over aversive stimuli and its relationship to stress, Psychol. Bull. 80 (1973) 286-303.

[4] L. Basset, H.M. Buchanan-Smith, Effects of predictability on the welfare of captive animals, Appl. Anim. Behav. Sci. 102 (2007) 223-245.

[5] N. Burgener, M. Dehnhard, H. Hofer. M.L. East. Does anal gland scent signal identity in the spotted hyaena? Anim. Behav. 77 (2009) 707-715.

[6] M. Dehnhard, M. Clauss, M. Lechner-Doll, H.H.D. Meyer, R. Palme, Noninvasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites, Gen. Comp. Endocrinol. 123 (2001) 111-120.

[7] S.M. Dloniak, Socioendocrinology of spotted hyenas: patterns of androgen and glucocorticoid excretion in a unique social system, (2004) PhD. Dissertation. Michigan State University.

[8] M.L. East, H. Hofer, A. Türk, Functions of birth dens in spotted hyaenas (*Crocuta crocuta*), J. Zool. (Lond.) 219 (1989) 690-697.

[9] M.L. East, H. Hofer, Loud calling in a female-dominated society: I. Structure and composition of whooping bouts of spotted hyaenas, Crocuta crocuta, Anim. Behav. 42 (1991) 637-649.

[10]M.L. East, T. Burke, K. Wilhelm, C. Greig, H. Hofer, Sexual conflicts in spotted hyenas: male and female mating tactics and their reproductive outcome with respect to age, social status and tenure, Proc. R. Soc. B. 270 (2003) 1247-1254.

[11]M.L. East, O.P. Höner, B. Wachter, K. Wilhelm, T. Burke, H. Hofer, Maternal effects on offspring social status in spotted hyenas, Behav. Ecol. 20 (2009) 478–483.

[12]M. Ferin, Stress and the reproductive cycle, J. Clin. Endocrinol. Metab. 84 (1999) 1768–1774.

[13]S.M. Fox, D.J. Mellor, E.C. Firth, H. Hodge, C.R. Lawoko, Changes in plasma cortisol concentrations before, during and after analgesia, anaesthesia and anaesthesia plus ovariohysterectomy in bitches, Res Vet Sci. 57 (1994) 110-118.

[14]L.G. Frank, Social organization of the spotted hyaena (*Crocuta crocuta*). I. Demography, Anim. Behav. 34 (1986) 1500–1509.

[15]L.G. Frank, S.E. Glickman, P. Licht, Fatal sibling aggression, precocial development, and androgens in neonatal spotted hyaenas, Science 252 (1991) 702-704.

[16]K. Gärtner, D. Büttner, K. Döhler, R. Friedel, J. Lindena, I. Trauzschold, Stress response of rats to handling and experimental procedures, Lab. Anim. 14 (1980) 267-274.

[17]W. Golla, H. Hofer, M.L. East 1999. Within-litter sibling aggression in spotted hyaenas: effect of maternal nursing, sex and age, Anim. Behav. 58 (1999) 715-726.

[18]W. Goymann, E. Möstl, T. Van’t Hof, M.L. East, H. Hofer, Noninvasive Fecal Monitoring of Glucocorticoids in Spotted Hyenas, *Crocuta crocuta*, Gen. Comp. Endocrinol. 114 (1999) 340-348.

[19]W. Goymann, M.L. East, B. Wachter, O. Höner, E. Möstl , T.J. Van’t Hof, H. Hofer, Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyaenas, Proc. R. Soc. Lond. B 268 (2001) 2453-2459.

[20]W. Goymann, J.C. Wingfield, Allostatic load, social status and stress hormones: the costs of social status matter, Anim. Behav. 67 (2004) 591-602.

[21]M. Heistermann, R. Palme, A. Ganswindt, Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis, Am. J. Primatol. 68 (2006) 257-273.

[22]H. Hofer, M.L. East, The commuting system of Serengeti spotted hyaenas: How a predator copes with migratory prey. I. Social organisation, Anim. Behav. 46 (1993a) 547-557.

[23]H. Hofer, M.L. East, The commuting system of Serengeti spotted hyaenas: How a predator copes with migratory prey. II. Intrusion pressure and commuters' space use, Anim. Behav. 46 (1993b) 559-574.

[24]H. Hofer, M.L. East, The commuting system of Serengeti spotted hyaenas: How a predator copes with migratory prey. III. Attendance and maternal care, Anim. Behav. 46 (1993c) 575-589.

[25]H. Hofer, M.L. East, Skewed offspring sex ratios and sex composition of twin litters in Serengeti spotted hyaenas (*Crocuta crocuta*) are a consequence of siblicide, Appl. Anim. Behav. Sci. 51 (1997) 307-316.

[26]H. Hofer, M.L. East, Biological conservation and stress, Adv. Stud. Behav. 27 (1998) 405-525.

[27]H. Hofer, M.L. East, Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective, Evol. Ecol. 17 (2003) 315-331.

[28]H. Hofer, M.L. East, Siblicide in Serengeti spotted hyenas: a long-term study of maternal input and cub survival, Behav. Ecol. Sociobiol. 62 (2008) 341-351.

[29]H. Hofer M.L. East, Stress and immunosuppression as factors in the decline and extinction of wildlife populations: the concepts, the evidence and the challenges In press: Aguirre AA, Daszak P, Ostfeld RS (eds) Conservation medicine: applied cases of ecological health, pp 82-107, Oxford University Press, New York, 2012.

[30]J. R. Ingram, J. N. Crockford and L. R. Matthews, Ultradian, circadian and seasonal rhythms in cortisol secretion and adrenal responsiveness to ACTH and yarding in unrestrained red deer (*Cervus elaphus*) stags, J. Endocrinol. 162 (1999) 289-300.

[31]M. Z. Khan, J. Altmann, S.S. Isani and J. Yu, A matter of time: evaluating the storage of fecal samples for steroid analysis, Gen. Comp. Endocrinol. 128 (2002) 57-64.

[32]S. M. Korte, G.A.H. Bouws, B. Bohus, Central actions of corticotropin-releasing hormone (CRH) on behavioral, neuroendocrine, and cardiovascular regulation: Brain corticoid receptor involvement, Horm. Behav. 27 (1993) 167-183.

[33]P. Kretzschmar, U. Ganslosser, and M. Dehnhard, The development of a non-invasive method to analyse the effect of season, mating behaviour and fighting on gonadal activity of male white rhinoceros (*Ceratotherium simum simum*) in South Africa, Horm. Behav. 45 (2004) 1-9.

[34]H. Kruuk, The Spotted Hyena, A Study of Predation and Social Behavior. Chicago: University of Chicago Press. 1972.

[35]M. Lepschy, C. Touma, R. Hruby, R. Palme. Noninvasive measurement of adrenocortical activity in male and female rats, Lab. Anim. 41 (2007) 372-387.

[36]R. M. Liprap, Stress and reproduction in domestic animals, Ann. N.Y. Acad. Sci. 697 (1993) 275-284.

[37]J. M. Mateo, Developmental and geographic variation in stress hormones in wild Belding's ground squirrels (*Spermophilus beldingi*), Horm. Behav. 50 (2006) 718-725.

[38]J. M. Mateo, Inverted-U shape relationship between cortisol and learning in ground squirrels, Neurobiol Learn Mem. 89 (2008) 582-590.

[39]S. L. Monfort, J.L. Brown, D.E. Wildt, Episodic and seasonal rhythms of cortisol secretion in male Eld’s deer (*Cervus eldi thamin*), J. Endocrinol. 138 (1993) 41-49.

[40]A. Munck, P.M. Guyre, N.J. Holbrook, Physiological functions of glucocorticoids in stress and their relation to pharmacological actions, Endocr. Rev. 5 (1984) 25-44.

[41]R. Palme, E. Möstl, Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood, Int. J. Mammal. Biol. 62 (1997), Suppl. II, 192-197.

[42]R. Palme, S. Rettenbacher, C. Touma, S.M. El-Bahr, E. Möstl, Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples, Ann. N.Y. Acad. Sci. 1040 (2005) 162-171.

[43]G.H. Pournelle, Observations on the birth and early development of the spotted hyaena, J. Mammal. 46 (1965) 503.

[44]C.R. Pryce, R. Palme, J. Feldon, Development of pituitary-adrenal endocrine function in the marmoset monkey: infant hypercortisolism is the norm, J. Clin. Endocrinol. Metab. 87 (2002) 691-699.

[45]S. Rettenbacher, E. Möstl, R. Hackl, K. Ghareeb, R. Palme, Measurement of corticosterone metabolites in chicken droppings, Br. Poult. Sci. 45 (2004) 704-711.

[46]M.L. Romero, Physiological stress in ecology: lessons from biomedical research, Trends Ecol. Evolut. 19 (2004) 249-255.

[47]M.L. Romero, J.M. Reed, Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp. Biochem. Physiol. A 140 (2005) 73-79.

[48]R.M. Sapolsky, The endocrine stress-response and social status in the wild baboon, Horm. Behav. 16 (1982) 279-292.

[49]R.M. Sapolsky, Stress, the aging brain, and the mechanisms of neuron death, MIT Press. 1992.

[50]S. Schatz, R. Palme, Measurement of faecal cortisol metabolites in cats and dogs: A non-invasive method for evaluating adrenocortical function, Vet. Res. Commun. 25 (2001) 271-287.

[51]L. Smale, L.G. Frank, K.E. Holekamp, Ontogeny of dominance in free-living spotted hyaenas: juvenile rank relations with adult females and immigrant males, Anim. Behav. 46 (1993) 467-477.

[52]C. Touma, N. Sachser, E. Möstl, R. Palme, Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice, Gen. Comp. Endocrinol. 130 (2003) 267-278.

[53]C. Touma, R. Palme, Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation, Ann. N.Y. Acad. Sci. 1046 (2005) 54-74.

[54]P.E. Van Meter, J.A. French, S.M. Dloniak, H.E. Watts, J.M. Kolowski, K.E. Holekamp, Fecal glucocorticoids reflect socio-ecological and anthropogenic stressors in the lives of wild spotted hyenas, Horm. Behav. 55 (2009) 329-337.

[55]C.C. Voigt, M. Fassbender, M. Dehnhard, K. Jewgenow, G. Wibbelt, H. Hofer, G.A. Schaub, Validation of a minimally invasive blood sampling technique for hormonal analysis in domestic rabbits, Gen. Comp. Endocrinol. 135 (2004) 100-107.

[56]D. von Holst, The concept of stress and its relevance for animal behavior, Adv. Study Behav. 27 (1998) 1-109.

[57]H. Wada, Glucocorticoids: Mediators of vertebrate ontogenetic transitions, Gen. Comp. Endocrinol. 156 (2008) 441-453.

[58]S.K. Wasser, K. Bevis, G. King, E. Hanson, Noninvasive physiological measures of disturbance in the northern spotted owl, Conserv. Biol. 11 (1997) 1019-1022.

[59]S. K. Wasser, K. E. Hunt, J.L. Brown, K. Cooper, C.M. Crockett, U. Bechert, J.J. Millspaugh, S. Larson, S.L. Monfort, A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species, Gen. Comp. Endocrinol. 120 (2000) 260-275.

[60]J.M. Weiss, Effects of coping responses on stress, J. Comp. Physiol. Psychol. 65 (1968) 251-260.

[61]J.C. Wingfield, P. Deviche, S. Sharbaugh, L.B. Astheimer, R. Holberton, R. Suydam, K. Hunt, Seasonal changes of the adrenocortical responses to stress in redpolls, *Acanthis flammea*, in Alaska, J. Exp. Zool. 270 (1994) 372-380.

[62]K.M. Young, J.L. Brown, K.L. Goodrowe, Characterization of reproductive cycles and adrenal activity in the black-footed ferret (*Mustela nigripes*) by fecal hormone analysis, Zoo Biol. 20 (2001) 517-536.

[63]K.M. Young, S.L. Walker, C. Lanthier, W.T. Waddell, S.L. Monfort, J.L. Brown, Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses, Gen. Comp. Endocrinol. 137 (2004) 148-165.

FIGURE CAPTIONS

Figure 1 HPLC profiles of radiolabelled cortisol metabolites in cpm (grey line) and cortisol immunoreactive substances in pg/ 20 µl (black line) analysed with the cortisol-3-CMO assay. Arrows indicate elution positions of cortisol, corticosterone, testosterone and dihydrotestosterone standard (DHT; 5α-androstan-17α-ol-3-one). Elution positions are ordered from the highest to the lowest polarity.

Figure 2 Changes in faecal glucocorticoid metabolite concentrations (in ng/g of faecal matter) measured with the cortisol-3-CMO assay during an ACTH challenge experiment (200 IU ACTH) in a male (a) and a female (b) captive spotted hyena. The * indicates that the value exceeded mean + 3SD.

Figure 3 Changes in faecal glucocorticoid metabolite concentrations (in ng/g of faecal matter) measured with the cortisol-3-CMO assay before and after the anaesthesia of one captive male spotted hyena. Points represent single samples and * indicates that the value exceeded mean + 3SD. No faeces were produced on day 1.

Figure 4 Pairwise comparisons of the mean concentrations of faecal glucocorticoid metabolites (in ng/g of faecal matter) in individually known juvenile spotted hyenas (N = 39) in the Serengeti National Park, Tanzania in two age categories: juveniles < 6 months (left); the same juveniles when aged between 6 and 24 months (right).

Figure 1

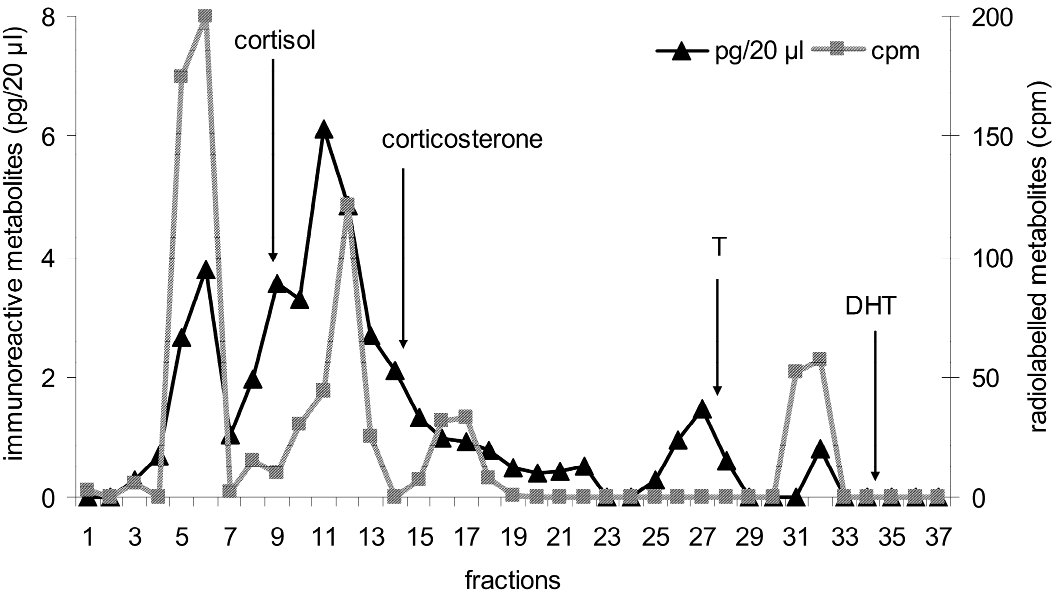

8
6
4
2
0
200
150
100
50
0
immunoreactive metabolites (pg/20 µl)
radiolabelled metabolites (cpm)
cortisol
corticosterone
T
DHT
pg/20 µl
cpm
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
fractions

Figure 2

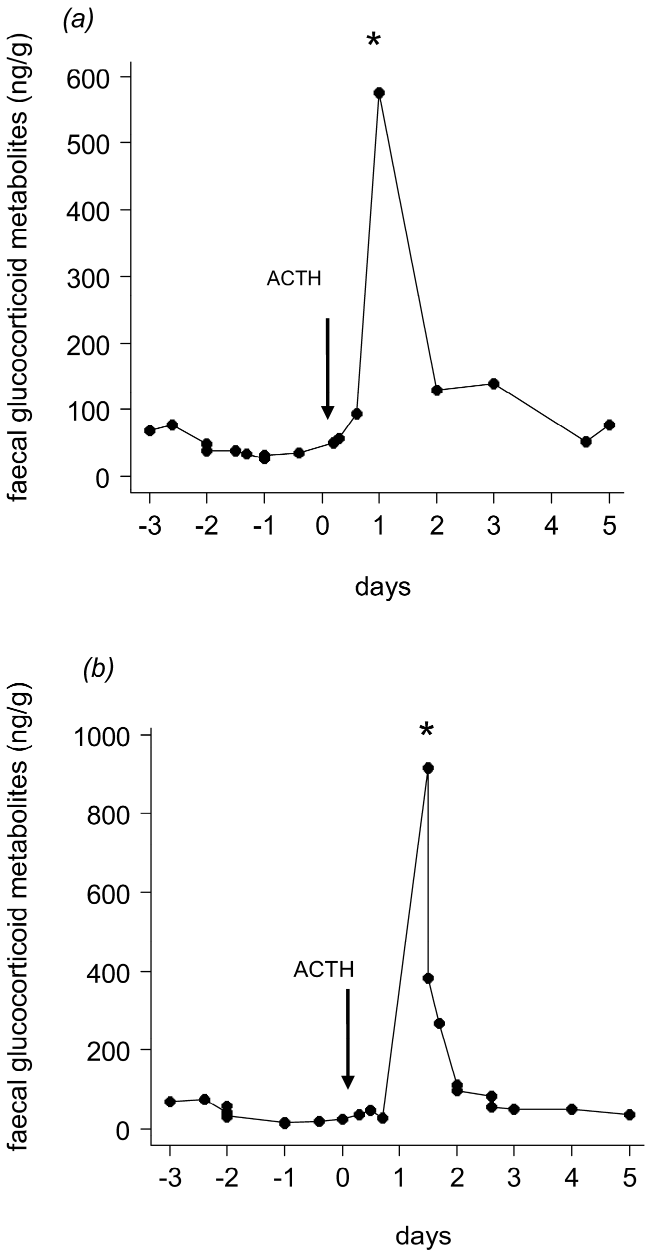

(a)
*
faecal glucocorticoid metabolites (ng/g)
600
500
400
300
200
100
0
ACTH
-3
-2
-1
0
1
2
3
4
5
days
(b)
*
faecal glucocorticoid metabolites (ng/g)
1000
800
600
400
200
0
ACTH
-3
-2
-1
0
1
2
3
4
5
days

Figure 3

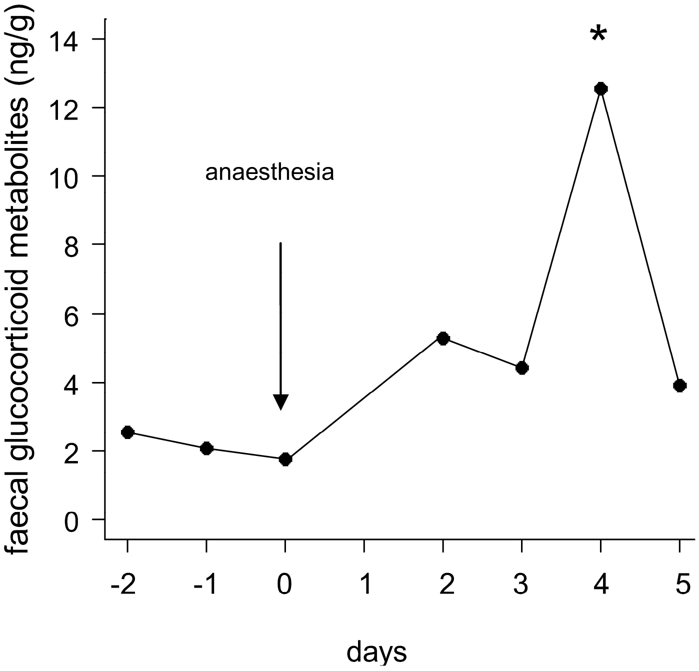
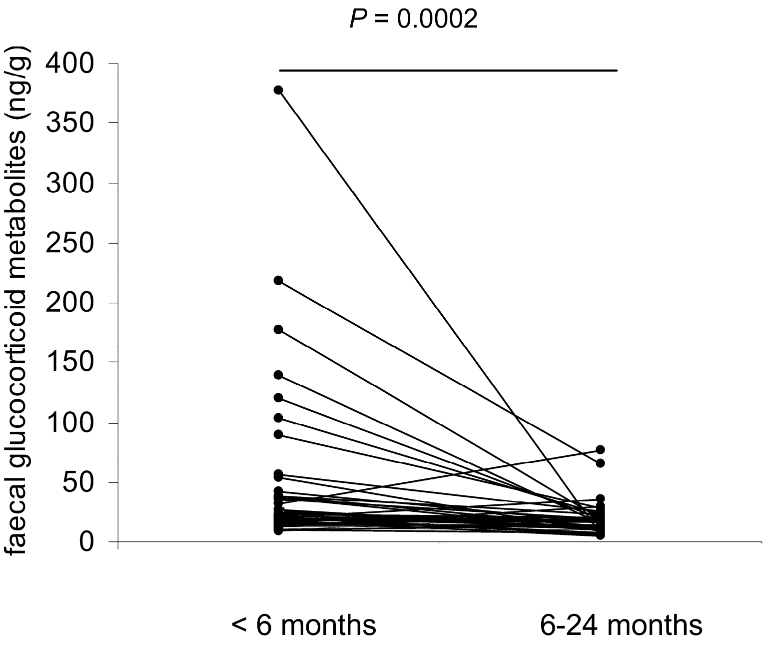

faecal glucocorticoid metabolites (ng/g)
0
2
4
6
8
10
12
14
anaesthesia
*
-2
-1
0
1
2
3
4
5
days

Figure 4


P = 0.0002
faecal glucocorticoid metabolites (ng/g)
400
350
300
250
200
150
100
50
0
< 6 months
6-24 months

CHAPTER 4

Sibling rivalry in spotted hyenas (*Crocuta crocuta*) is stressful

(manuscript)

SARAH BENHAIEM, MARTIN DEHNHARD, HERIBERT HOFER,

JANINE HELMS, MARION L. EAST

Author contribution:

The concept of this study was developed by Sarah Benhaiem (SB), Marion L. East (ME) and Heribert Hofer (HH). Faecal samples of Serengeti free-ranging spotted hyenas were collected by SB, ME and Janine Helms (JH) and faecal extraction was done by SB and JH. The development of the cortisol-3-CMO enzyme immunoassay and the measurement of faecal glucocorticoid metabolite concentrations were done by MD. Statistical analysis was done by SB, under guidance of HH. Writing of the manuscript was led by SB and ME, HH edited and commented the manuscript.

Sibling rivalry in spotted hyenas (*Crocuta crocuta*) is stressful

SARAH BENHAIEM, MARTIN DEHNHARD, HERIBERT HOFER, JANINE HELMS, MARION L. EAST

Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

Corresponding author: benhaiem@izw-berlin.de,

phone: +49-30-51-68-520

Abstract: Glucocorticoids are used as measures of physiological stress but their relation to intense sibling rivalry in free-ranging mammalian litters has not been investigated. We measured non-invasively faecal glucocorticoid metabolite (fGCM) concentrations in spotted hyena twin litters (80 siblings). In this facultative siblicidal species, early rivalry is intense when milk provisioning is low, leads to dominant and subordinate roles, and involves trained loser effects. We expected subordinates to experience more stress than dominants during their first six months of life when litters are entirely dependent on milk. Subordinates had nearly twice the fGCM concentrations of dominants. Dominant females had higher fGCM concentrations than dominant males, probably because subordinate siblings raised with a dominant female are behaviourally more challenging than subordinate siblings with a dominant male during periods of low milk provisioning rates. Our results confirm that intense sibling rivalry in this species is stressful during the critical age period and are consistent with the concept of allostatic load.

Key-words: faecal glucocorticoid metabolites, non-invasive method, sibling rivalry, spotted hyena, loser effect

1. INTRODUCTION

In species that display facultative siblicide, within-litter or brood dominance status can profoundly affect the fitness of competing siblings [1]. Dominant siblings aggressively skew access to food delivered by parents in their favour (e.g. [2,3,4]), thereby improving their growth and survival compared to their subordinate sibling [1,5,6,7]). In several bird species, subordinate chicks have higher levels of physiological stress than their dominant sibling [8], particularly when fed energy restricted diets (e.g. [9,10]). To our knowledge, the impact of sibling rivalry on the physiological stress levels of dominant and subordinates has not been investigated in free-ranging mammalian litters.

Glucocorticoids mobilise energy to cope with short-term environmental or social challenges [11]. Chronically high glucocorticoid levels can have a negative impact on fitness (see [12] for a review); they may lead to immunosuppression and atrophy of tissues, impaired cognition, or increase mortality risk [13]. The measurement of faecal glucocorticoid metabolites (fGCM) is a recent non-invasive tool to assess stress levels of vertebrates [12].

Here we present the first investigation of the impact of sibling rivalry on fGCM levels in free-ranging spotted hyena (*Crocuta crocuta*) twin litters that were entirely dependant on highly nutritious maternal milk [14]. Young subordinates exhibit trained loser effects and are excluded from maternal teats by their aggressive dominant siblings during suckling bouts [4]. When litters are completely dependent on milk during the first six month of life, dominants increase rates of aggression during suckling bouts as maternal input (milk) declines [15]. When maternal input is low, dominants can enforce starvation on their siblings, and this facultative siblicide typically occurs within the first three months of age [7,16]. For these reasons we expected stress levels measured in terms of fGCM concentrations to be higher in subordinates of spotted hyena twin litters than in dominants, when dependent on maternal milk. Because subordinates raised with a dominant sister are more assertive when hungry than subordinates with a dominant brother [4], we also predicted that dominant sisters should show higher fGCM concentrations than dominant brothers.

2. MATERIAL AND METHODS

(a) *Study population*

The study was conducted in the Serengeti National Park in Tanzania between 2007 and 2010 on three clans. Clans were stable multi-female, multi-male fission-fusion groups with separate linear dominance hierarchies among philopatric adult females and reproductively active, mostly immigrant adult males [14] that defended permanent territories where large, unpredictable fluctuations in prey abundance [17] occurred. When the density of migratory prey was high in a clan's territory, all lactating females were able to nurse their litters at the communal den regularly. When migratory prey were absent from the territory, all lactating mothers undertook frequent long distance foraging trips (40-70km) to the nearest concentrations of prey. All animals, including cubs, were individually recognised [15,17].

Communal dens were monitored for several hours around dawn and dusk. We classified a cub as the subordinate member of the litter if the number of submissive behaviours observed for that cub was greater than that recorded for its littermate [15]. The age of juveniles was estimated in days (accuracy ± 7 days) using characteristics described in [15]. Animals were considered adults at 24 months. Cub sex was determined at approximately three 3 months of age, using the dimorphic glans morphology of the erect phallus [18]. We measured fGCM in 80 siblings aged between 52 and 738 days, including 39 dominants (20 males and 19 females) and 41 subordinates (22 males and 19 females). Our within-litter comparison comprised 23 twin litters: 10 same-sex cubs (two female cubs: 4 litters, two male cubs: 6 litters) and 13 mixed-sex litters. In mixed-sexed litters, the dominant sibling was female in 8 litters and was male in 5 litters.

(b) *Processing of faecal samples*

We collected 191 faeces immediately after defaecation and stored them in the field in a cold box for less than 3 hours. Then, faeces were mechanically mixed and subsamples of faeces were stored in 5 ml tubes in liquid nitrogen or frozen at approximately -10 °C until transported frozen to Berlin where they were stored at - 80°C until analysed.

(c) *Faecal glucocorticoid metabolites*

To quantify the concentrations of glucocorticoid metabolites in each faeces, we used the cortisol-3-CMO enzyme immunoassay, validated for use in the spotted hyena [19]. High performance liquid chromatography analysis of faecal extracts revealed that glucocorticoids mainly comprised cortisol steroid metabolites detected by the antibody. Parallelism was demonstrated. Mean sensitivity of the assay at 90% binding was 0.5 pg per well. Each sample was assayed in duplicate. Intra- and inter-assay coefficients of variation were 16.5 % and 23.1% for a low and 5.3% and 10.1% for a high concentration pool. Final fGCM concentrations are expressed as ng/g faecal matter.

(d) ***Statistics***

Analyses were carried out using R (R Development Core Team, v. 2.11.1). The threshold for significance was at 5% and all tests were two-tailed. Statistics are quoted as means ± s.e.m. To test whether subordinates have higher fGCM concentrations than dominants when less than six months of age, we first applied a two-way ANOVA with fGCM concentrations as the response variable, and sibling status (two categories: dominant versus subordinate), age (two categories: less than six months vs. between six and 24 months) and the interaction between sibling status and age, as explanatory variables. We calculated mean fGCM for each individual for each age category to avoid pseudo-replication. If an individual contributed faecal samples to both age categories, we randomly selected one for the analysis. Because the response variable was not normally distributed we transformed it using a reciprocal square root transformation.

To increase robustness we tested the same prediction in the subsample of matched siblings. We compared with a Wilcoxon signed rank test, within-litter differences in fGCM concentrations using all faecal samples produced by both dominants and subordinates of the same litter before the age of six months. To test the effect of sex on fGCM concentrations of dominants we used an ANCOVA with concentrations of fGCM for dominants as response variable, dominant sex and subordinate sex as explanatory variables and fGCM concentrations of the subordinate as covariate. Dominants and subordinates fGCM concentrations were not normally distributed, thus were log-transformed to base 10. Residuals of all models were normally distributed (Lilliefors test) and variances showed no heterogeneity (residual plots).

3. RESULTS

Subordinates had significantly higher fGCM concentrations than dominants ($F_{1,76} = 5.428$; $p = 0.022$) across both age categories ($F_{1,76} = 1.507$; $p = 0.223$) and that increase depended on age ($F_{3,76} = 5.863$; $p = 0.018$, figure 1). Mean fGCM concentrations for subordinates less than six months of age were 34.5 ± 7.2 ng/g, for dominants 17.4 ± 1.7 ng/g.

Concentrations of fGCM in litters younger than six months of age were also significantly (Wilcoxon signed rank test: V = 64; n = 23 twin litters; $p = 0.023$) higher in matched pairs of subordinates (49.8 ± 13.7 ng/g) than in their dominant siblings (36.2 ± 16.4 ng/g). In matched sibling pairs, dominant females had significantly ($F_{1,19} = 6.629$; $p = 0.019$; figure 2) higher fGCM concentrations (56.3 ± 25.3 ng/g; n=12) than dominant males (14.3 ± 2.0 ng/g; n=11), and no effect of the subordinate’s sex ($F_{1,19} = 0.698$; $p = 0.414$), after accounting for the fGCM levels of the matched subordinate ($F_{1,19} = 4.791$; $p = 0.041$) was found.

4. DISCUSSION

Consistent with our prediction, young subordinate siblings in spotted hyena twin litters showed higher fGCM concentrations than dominants. Aggressive subordination [15] involving trained loser effects [4] and food deprivation [7,15] are likely to lead to elevated stress in subordinates. As expected, dominant females had higher fGCM concentrations than dominant males.

Sibling rivalry was demonstrated to be stressful to subordinates in avian broods (e.g. [20]) but its effect in mammalian litters is largely unknown. Siblings in experimentally enlarged captive guinea pig (*Cavia aperea f. porcellus*) litters showed increased cortisol concentrations when competing non-aggressively for maternal milk [21]. To our knowledge this is the first study to test the effect of within-litter dominance on sibling glucocorticoid levels in a free-ranging mammalian species. The higher stress level of subordinates during the period when litters entirely depend on maternal milk, is probably a consequence of social subordination during several months of intense rivalry for access to milk, high rates of aggression from dominants [15] and trained loser effects [4]. Compared to other spotted hyena populations [22], this is exacerbated in our study population by the periodical need of both siblings to mobilise energy during protracted periods of several days while mothers are absent foraging [4,7,15,23] and the associated increase in aggression and attempted monopolisation of milk by the dominant [4,15]. In extreme cases, monopolisation is so successful that the subordinate will starve to death (facultative siblicide, [7]). We would therefore expect siblings in other populations to display lower fGCM concentrations.

Dominants should show higher glucocorticoid levels than subordinates when dominance is unstable and acquired or maintained at a high cost [24,25]. In such systems, subordinates behaviourally challenge dominants and dominants experience a high allostatic load, i.e. a high cost of maintaining homeostatis, resulting in elevated levels of glucocorticoids [11]. In spotted hyena litters dominant sisters are more strongly challenged by their hungry subordinate siblings than dominant brothers [4], possibly because subordinates with dominant sisters are more at risk of facultative siblicide [4,16]. In accordance with this result, dominant females had four times higher fGCM concentrations than dominant males. Similarly, dominant blue-footed booby chicks which used to live with a submissive subordinate broodmate and were experimentally paired with (non-submissive) singleton chicks showed a marked increase in corticosterone concentrations [20].

In conclusion, elevation of glucocorticoid levels in young spotted hyena siblings is the result of several processes; including social subordination, extended periods of maternal absence and sustained challenges of dominants by subordinates.

ACKNOWLEDGEMENTS

We thank the Tanzania Commission for Science and Technology for permission to conduct the study, TAWIRI and TANAPA for their support of the research. We thank A. Francis, T. Shabani in Tanzania and D. Thierer and K. Wilhelm in Berlin for their assistance and the IZW for funding.

REFERENCES

1 Mock, D.W. & Parker, G. A. 1997 *The Evolution of Sibling Rivalry*. Oxford: Oxford University Press.

2 Drummond, H. & Garcia Chavelas, C. 1989 Food shortage influences sibling aggression in the blue-footed booby. *Anim. Behav*. **37**, 806–819. (doi: 10.1016/0003-3472(89)90065-1)

3 Cook, M.I., Monaghan, P. & Burns, M.D. 2000 Effects of short-term hunger and competitive asymmetry on facultative aggression in nestling black guillemots *Cepphus grylle*. *Behav. Ecol.* **11**, 282–287. (doi: 10.1093/beheco/11.3.282)

4 Benhaiem, S., Hofer, H., Kramer-Schadt, S. & East, M.L. 2012a Sibling rivalry: training effects, emergence of dominance and incomplete control (in revision)

5 Fraser, D. & Thompson, B.K. 1991 Armed sibling rivalry among suckling piglets. *Behav. Ecol. Sociobiol*. **29**, 9-15. (doi: 10.1007/BF00164289).

6 Bonabeau, E., Deneubourg, J.L. & Theraulaz, G. 1998 Within-brood competition and the optimal partitioning of parental investment. *Am. Nat*. **152**, 419-427. (doi: doi:10.1086/286179)

7 Hofer, H. & East, M.L. 2008 Siblicide in Serengeti spotted hyenas: a long-term study of maternal input and cub survival. *Behav. Ecol. Sociobiol.* **62**, 341-351. (doi:10.1007/s00265-007-0421-3)

8 Tarlow, E.M., Wikelski M. & Anderson, D. 2001 Hormonal correlates of siblicide in Galápagos Nazca boobies. *Horm. Behav.* **40**, 14-20. (doi:10.1006/hbeh.2001.1661)

9 Nuñez de la Mora, A., Drummond, H. & Wingfield, J. 1996 Hormonal correlates of dominance and starvation-induced aggression in chicks of the Blue-footed Booby. *Ethology* **102**,748-761. (doi: 10.1111/j.1439-0310.1996.tb01164.x)

10 Kitaysky, A.S., Piatt, J. F., Wingfield J. C & Romano, M. 1999 The adrenocortical stress-response of Black-legged Kittiwake chicks in relation to dietary restrictions. *J. Comp. Physiol. B* **169**, 303-310. (doi: 10.1007/s003600050225).

11 Goymann, W. & Wingfield, J.C. 2004 Allostatic load, social status and stress hormones: the costs of social status matter. *Anim. Behav.* **67**, 591-602. (doi: 10.1016/j.anbehav.2003.08.007).

12 Hofer, H. & East, M.L. 2012 *Stress and immunosuppression as factors in the decline and extinction of wildlife populations: the concepts, the evidence and the challenges* In Conservation medicine: applied cases of ecological health (ed. Ostfeld RS), pp 82-107. New York: Oxford University Press.

13 Pride, R.E. 2005 High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). *Biol. Lett.* **1**,60-63. (doi: 10.1098/rsbl.2004.0245)

14 Hofer, H. & East, M.L. 1995 *Population dynamics, population size and the commuting system of Serengeti spotted hyenas*. In Serengeti II. Dynamics, Management, and Conservation of an *Ecosystem (ed. A.R.E. Sinclair, P. Arcese)*, pp. 332-363. Chicago: University of Chicago Press.

15 Golla, W., Hofer, H. & East, M.L. 1999 Within-litter sibling aggression in spotted hyaenas: effect of maternal nursing, sex and age. *Anim. Behav.* **58**, 715-726. (doi:10.1006/anbe.1999.1189)

16 Hofer, H. & East M.L. 1997 Skewed offspring sex ratios and sex composition of twin litters in Serengeti spotted hyaenas (*Crocuta crocuta*) are a consequence of siblicide. *Appl. Anim. Behav. Sci.* **51**, 307–316. (doi: 10.1007/s00265-007-0421-3).

17 Hofer, H. & East, M.L. 1993a The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. I. Social organization. *Anim. Behav.* **46**, 547–557. (doi:10.1006/anbe.1993.1222)

18 Frank, L.G., Glickman, S.,E. & Licht, P. 1991 Fatal sibling aggression, precocial development, and androgens in neonatal spotted hyaenas. *Science* **252,** 702–704. (doi: 10.1126/science.2024122)

19 Benhaiem, S., Dehnhard, M., Bonanni, R., Hofer, H., Goymann, W., Eulenberger, K. & East, M.L. 2012b Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in spotted hyenas *Crocuta crocuta* (minor revisions)

20 Ramos-Fernandez, G., Nunez-de la Mora, A., Wingfield, J. C. & Drummond H. 2000. Endocrine correlates of dominance in chicks of the blue-footed booby (*Sula nebouxii*): Testing the challenge hypothesis. *Ethol. Ecol. Evol.* **12,** 27–33.

21 Fey, K. & Trillmich, F. 2008 Sibling competition in guinea pigs (*Cavia aperea f. porcellus*): scrambling for mother’s teats is stressful. *Behav. Ecol. Sociobiol.* **62**, 321–329. (doi: 10.1007/s00265-007-0419-x)

22 Wachter, B., Höner, O.P., East, M.L., Golla, W. & Hofer, H. 2002 Low aggression levels and unbiased ratios in a prey-rich environment: no evidence of siblicide in Ngorongoro spotted hyenas (*Crocuta crocuta*). *Behav. Ecol. Sociobiol.* **52**, 348–356. (doi: 10.1007/s00265-002-0522-y).

23 Hofer, H. & East, M.L. 1993c The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. III. Attendance and maternal care. *Anim. Behav.* **46**, 575–589.

24 Goymann, W., East, M.L., Wachter, B., Höner, O.P., Möstl, E., Van’t Hof, T.J. & Hofer, H. 2001 Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyaenas. *Proc. R. Soc. Lond. B* **268**, 2453-2459. (doi: 10.1098/rspb.2001.1828)

25 Gesquiere, L.R., Learn, N.H., Simao, M.C.M., Onyango, P.O., Alberts, S., Altmann, J. 2011 Life at the top: rank and stress in wild male baboons. *Science* **333**, 357-360. (doi: 10.1126/science.1207120)

FIGURE CAPTIONS

Figure 1 Faecal glucocorticoid metabolite concentrations (ng/g) of dominant (dom) and subordinate (sub) spotted hyena siblings when less than six months and when between six and twenty-four months. Bars represent means ± s.e.m. and numbers above sample sizes.

Figure 2 Faecal glucocorticoid metabolite concentrations (ng/g) of dominant males and dominant females when less than six months. Bars represent means ± s.e.m. and numbers above sample sizes.

Figure 1

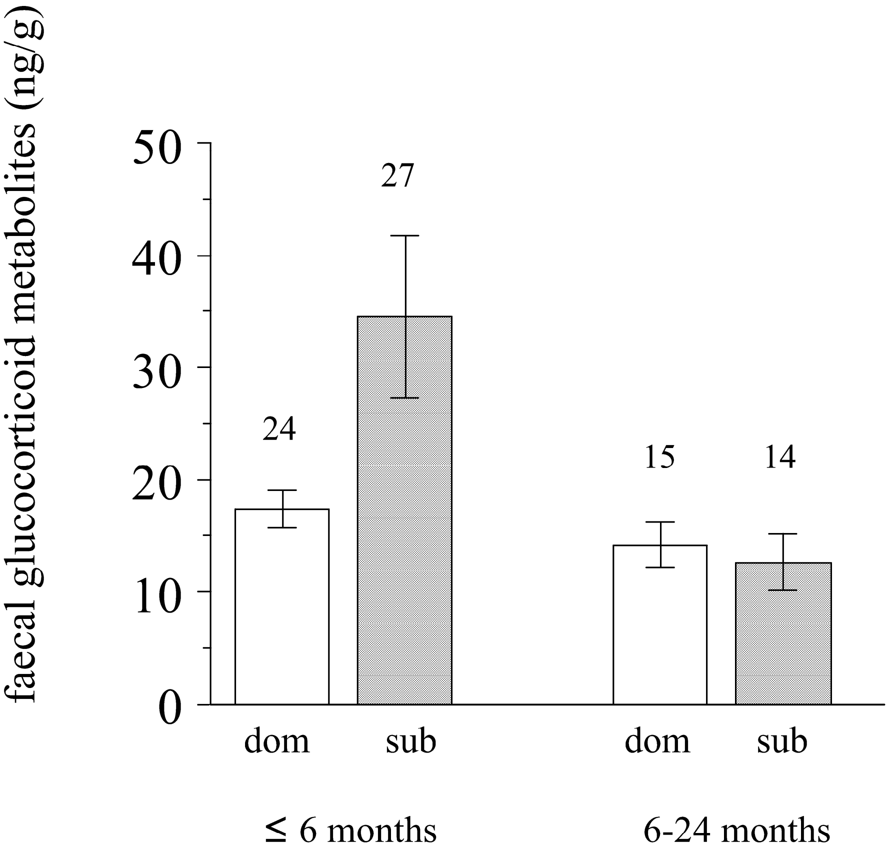
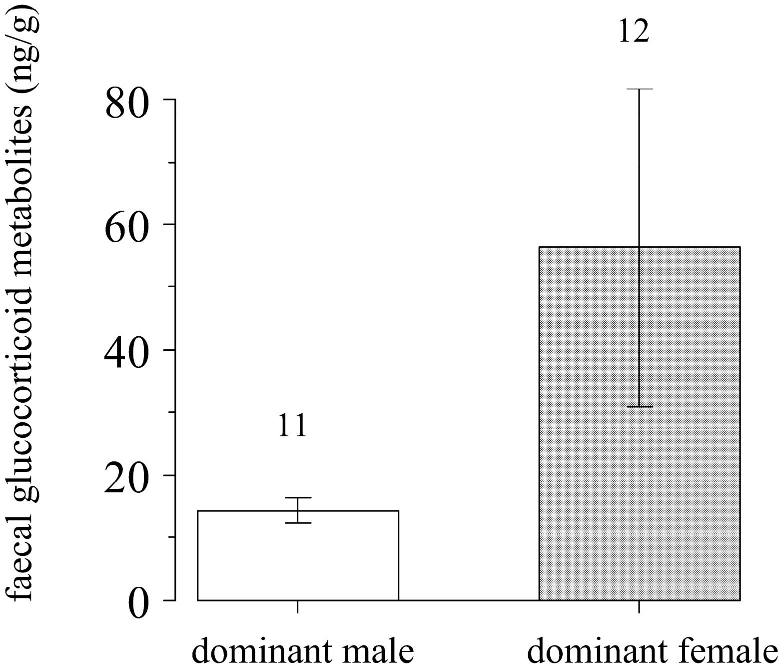

faecal glucocorticoid metabolites (ng/g)
50
40
30
20
10
0
27
24
15
14
dom
sub
dom
sub
≤ 6 months
6-24 months

Figure 2


faecal glucocorticoid metabolites (ng/g)
80
60
40
20
0
11
12
dominant male
dominant female

CHAPTER 5: GENERAL DISCUSSION

Siblings may compete intensively for food resources provided by their parents (Mock and Parker 1997). This rivalry can be asymmetrical because siblings may differ in their competitive abilities. In many bird species, asymmetries are conferred by hatching asynchrony, a process by which the first-hatched chick obtains a competitive advantage relative to later-hatched chicks. Differential maternal investment in egg size (reviewed by Williams 1994) or egg constituents, such as androgens or nutrients (Groothuis et al. 2005), may further influence the development of competitive asymmetries between siblings. Sibling rivalry may also be asymmetrical between the sexes. For example, in sexually dimorphic mammalian species such as domestic sheep (Burfening 1972) and Soay sheep (*Ovies aries*; Korsten et al. 2009), androgens produced by male foetuses may negatively influence the development of co-twin female siblings and reduce their birth weight. In these species, the initial competitive disadvantage of females born with a male co-twin had important fitness consequences in that it reduced their future reproductive success and survival when compared to that of females born with co-twin sisters. In (at least) one bird species, the blue-footed booby, behavioural trained winner and loser effects reinforce initial competitive asymmetries between siblings, and lead to the establishment of a stable dominance relationship within a brood (Drummond and Canales 1998). In summary, asymmetrical sibling rivalry may result in dominant siblings obtaining a greater share of parentally provided food resources than subordinate siblings.

Siblings may compete non-aggressively through begging, or use aggressive tactics to increase their share of parental provided food (or both) (Mock and Parker 1997). The spotted hyena is one of the few mammalian species in which sibling rivalry can be intense and may lead to facultative siblicide when ecological conditions cause maternal input to decrease below the level required to sustain the entire twin or triplet litter (Hofer and East 2008). In this species, rivalry is most intense when siblings compete for access to maternal milk (Golla et al. 1999). Variation in ecological conditions (i.e., prey abundance) determines the intensity of sibling rivalry and the occurrence of facultative siblicide (Golla et al. 1999, Hofer and East 2008). Spotted hyenas in the Ngorongoro Crater, in Tanzania, feed on high and relatively constant levels of preys throughout the year (Wachter et al. 2002). Lactating females in the Crater visit the communal den and nurse their offspring every day or every second day, resulting in low rates of sibling aggression and an absence of facultative siblicide (Wachter et al. 2002).

In contrast, in the Serengeti National Park, or in the Kalahari desert in southern Africa (Mills 1990), clan members feed on significantly lower and fluctuating levels of prey. In the Serengeti National Park, the decline in prey abundance in a clan territory may cause mothers to decrease the frequency at which they visit the communal den to nurse their offspring. As the total level of maternal input available to a twin litter decreases, sibling rivalry intensifies, and facultative siblicide may occur (Hofer and East 2008). As unpredictable variation in food availability significantly shapes the evolution of sibling rivalry (Mock and Parker 1997), spotted hyena twin litters reared in the Serengeti National Park are ideal to investigate the behavioural tactics of competing siblings. Within-litter dominance in Serengeti spotted hyena litters has a major influence on a young spotted hyena's life history because it significantly affects its growth rate and survival to adulthood (Hofer and East 1997, 2008). It is in this context that my thesis aimed to study in detail sibling rivalry in the spotted hyena.

The first aim of my thesis was to investigate whether several behavioural mechanisms predicted by "bird models" of sibling rivalry applied to competing spotted hyena siblings. For this purpose, I studied sibling interactions during rivalry for access to teats (chapter 2); in particular the tactics used by dominant siblings to achieve control over access to both functioning maternal teats, and the counter-tactics used by subordinate siblings to resist it. I found that dominant siblings skewed access to maternal milk in their favour during rivalry, and that this skew was influenced by several key factors: "desperado" behaviours by subordinates, hunger, age, and sex. In this chapter, I also investigated the behavioural mechanism likely to be responsible for the emergence of a dominance relationship between spotted hyena siblings, and demonstrated for the first time the role of trained effects in young spotted hyena litters.

The second focus of the study was on steroid hormones and aimed at measuring the impact of intense sibling rivalry on the level of physiological stress in both siblings. In chapter 3, I detailed the development and validation procedure of a non-invasive method for the measurement of glucocorticoid metabolites in the faeces of spotted hyenas. The second aim of chapter 3 was to apply this method to investigate for the first time changes in levels of glucocorticoids during the juvenile period. I found that juveniles were more physiologically stressed when young than when older. Previous studies suggested that a low social status may be physiologically stressful in adults (Abbot et al. 2003, Goymann and Wingfield 2004), and in subordinate chicks (Nunez de la Mora et al. 1996, Tarlow et al. 2001). The aim of chapter 4 was to examine whether within-litter dominance and sex influenced the faecal glucocorticoid metabolite levels of both dominant and subordinate siblings. During the most intense period of sibling rivalry, subordinate siblings had higher physiological stress levels than dominants, and dominant sisters had higher physiological stress levels than dominant brothers. Here, I aim to outline the implications of the results presented in chapters 2 to 4 and discuss why they provide us with a better understanding of intense sibling rivalry in a species with facultative siblicide.

5.1 Behavioural mechanisms of sibling rivalry in the spotted hyena

5.1.1 The control over access to maternal milk: taken by force or gained by consensus?

As opposed to human and potentially some non-human primate species (e.g., brown capuchin monkeys (*Cebus appella*); Brosnan and de Waal. 2003) showing developed food sharing and co-operative behaviours, social relationships among adult spotted hyenas are likely to be more selfish, at least regarding food sharing. Aggressive sibling competition for milk seems to be in accordance with the lack of obvious altruistic behaviours observed among feeding adults. Previous studies on sibling rivalry in the spotted hyena focused on factors influencing the intensity of sibling rivalry (Smale et al. 1995, Drea et al. 1996, Golla et al. 1999, Wachter et al. 2002). In habitats in which there is extreme fluctuations in prey abundance, the ecological and social factors that ultimately lead to facultative siblicide in this species have also been investigated, including the fitness benefits obtained by dominant members of litters that committed siblicide (Hofer and East 1997, 2008).

In chapter 2, I aimed at considering the neglected role of the subordinate sibling during conflicts for access to maternal milk; i.e., the influence of its behaviours on the dynamics of sibling interactions. In contrast to senior chicks, dominant spotted hyena siblings which seek to skew maternal milk supply in their favour have to trade-off suckling and aggression (Hudson and Trillmich 2008). My results reveal that dominant spotted hyena siblings benefit from their investment in aggression (at least during the first 15 minutes of a suckling bout) – in particular when litters are young (less than 6 months of age). Indeed, during each aggressive act, dominants lose little suckling time (approximately 3 seconds per aggressive action), but achieve to exclude their subordinate siblings from milk for a significantly and consistently longer period (approximately 10 seconds per aggressive action; chapter 2, supplementary figure 1 in electronic supplementary material 2). The suckling time lost by the subordinate after each aggressive act initiated by the dominant corresponded to the lost opportunity of obtaining energy equivalent to 6.37kJ. Thus, should the aggression rate by dominants remain relatively high during a suckling period of one hour, which is the average duration of a suckling period when a mother returns to the communal den (chapter 2), the subordinate would lose considerably more suckling time than the dominant and would probably ingest less milk than the dominant during a suckling period. Indeed, previous research in the Serengeti National Park (Hofer and East 1997, 2008) and in our study in chapter 2 indicated that when siblings are completely dependent on maternal milk, dominants have higher growth rates than their subordinate siblings. This indicates that dominant siblings take a significantly larger share of the (fixed) milk input provided by the mother to her twin litter (demonstrated in Hofer and East 2008). Thus, my results suggest that aggression by dominant siblings during suckling bouts is the tactic by which dominants skew access to milk in their favour in terms of lost suckling times, and possibly in terms of milk consumption by each sibling.

Although dominant siblings in almost all litters significantly skewed access to milk in their favour, my results suggest that they did not completely control access to maternal teats. Indeed, when hungry, subordinates were less submissive in response to aggression than when fed (chapter 2), decreasing the relative delay between siblings (chapter 2, figure 1a,b) and their own lost suckling time (chapter 2, figure 3b), and increasing the suckling time lost by dominants (in litters of specific sex compositions, chapter 2, figure 2d). In osprey (*Pandion haliaetus*) broods, the aggression by senior chicks was adjusted in relation to the availability of food (Poole 1982, Forbes 1989, Machmer and Ydenberg 1998). Senior chicks were more aggressive when hungry and thereby increased their share of food items delivered by their parents (Machmer and Ydenberg 1998). Hence, senior chicks relatively enhanced their feeding advantage through increased aggression. This phenomenon has also been demonstrated among cattle egret (Fujioka 1985, Ploger and Mock 1986) and blue-footed booby (Drummond et al. 1986) broods and it suggests that senior chicks had absolute control over the food that their junior broodmate(s) accessed.

In spotted hyena litters, dominant siblings also increased the intensity and rate of aggression when the level of maternal input in terms of milk decreased (Golla et al. 1999). However, in contrast to the bird studies described above, my results suggest that dominant siblings achieved the highest skew when not hungry (chapter 2, figure 1b). In hungry spotted hyena litters, increased aggression by dominants and increased assertiveness by subordinates led to a relative decrease in the skew in favour of dominants in terms of access to teats. In addition, through increased assertiveness, subordinate (females) increased the suckling time lost by their dominant siblings during conflicts. In 7 of 97 focal samples, hungry subordinates lost less suckling time per aggressive action than dominants. Subordinates were also able to reverse the dominance order in approximately 7% of all litters. Together, these results showed that dominants may fail to skew access to maternal milk in their favour in some situations. In addition, subordinates were able to reduce the skew in favour of dominants by being less submissive during conflicts. Thus, my results show that spotted hyena siblings do not exert an absolute level of control over their subordinate sibling's access to resources (milk), in contrast to what is assumed (Parker et al. 1989, Mock and Parker 1997) and documented (Mock and Parker 1997, Drummond 2006) in many bird species.

The "desperado sibling hypothesis" proposed by Drummond et al. (2003), inspired by Grafen's (1987) theoretical formulation of the "desperado effect", predicts extreme aggressiveness in subordinate chicks that have near zero prospects of survival. More recently, Drummond (2006) suggested that in species with facultative siblicide, subordinate siblings should increase their level of resistance against dominants as their survival prospects diminish. The results presented in chapter 2 strongly support the idea that subordinates do decrease their level of submissiveness in response to their dominant siblings when their survival prospects (their "cost of subordination"), measured in terms of their hunger level, were diminished. My results suggest that it is partly the subordinate's "acceptance" (or rejection) of subordination that determines the degree of control achieved by dominants over the access to maternal teats (also see analysis using the variable *mscore* in chapter 2, electronic supplementary material 3). This would be consistent with some of the first explorations of the concept of dominance. For example, dominance should not be defined by aggressive behaviour (Rowell 1974, Bernstein 1981, Drews 1993) and is not necessarily correlated with aggression (Bernstein 1981, Drews 1993). Instead, dominance relationships are best defined by the submissiveness of one individual rather than the aggressiveness of the other (e.g., Rowell 1974). My results further suggest that behavioural conventions are used to express dominance relationships between spotted hyena siblings (i.e., when subordinates respond with submission to low aggression by dominants) provided food is plentiful and thus acceptance of a subordinate role does not entail an elevated cost in terms of reduced milk intake or survival (Drummond 2006). Such behavioural conventions may be established during trained winner and loser effects (Drummond and Canales 1998).

5.1.2 Trained winner and loser effects

The positive or negative experiences of winning or losing conflicts may modify the perception by contestants of their own fighting ability (Hsu et al. 2006, Fawcett and Johnstone 2010). As a result, winning an encounter trains an individual to increase its chance to win the next encounter, and conversely, losing an encounter trains an individual to increase its chance to lose the next encounter (Chase et al. 1994). We found compelling evidence for a process akin to a trained loser effect in young spotted hyena twin litters (chapter 2, figure 4). Here, such training required less than 15 minutes but had a long-term effect on the dominance relationship between littermates (see next section). Trained loser effects were evident in litters less than three months of age, in which the subordinate sibling increased its level of submission in response to repeated aggressive actions. This indicates that during the first interactions of a suckling bout when a mother starts nursing her twin litter, subordinate siblings are at first assertive (or aggressive) in response to aggression by dominants, and that they become increasingly submissive in response to a series of aggressive acts by their dominant siblings. Consistent with a trained loser effect in litters less than 3 months of age, the degree of skew in suckling time achieved by dominant littermates (chapter 2, figure 2c) and the suckling time lost by subordinates were most extreme when litters were young (chapter 2, figure 3c).

My results suggest that the most intense period of training occurs when spotted hyena siblings are young, typically less than three months of age. After this period of intense training, between three and six months of age, subordinates immediately showed submission in response to the first aggressive acts of the dominant at the beginning of a suckling bout (unpublished data). It is worth reminding that during suckling bouts, dominant siblings show the highest intensity of aggression during the first three months of age, and that the rate of aggression by dominant siblings decreases linearly throughout the first six months of age (Golla et al. 1999). Hence, in older litters, lower levels of aggression by dominant siblings seem to be sufficient to induce submission in subordinates. A similar result was found in blue footed booby broods (Drummond and Canales 1998, Valderrábano-Ibarra et al. 2007). This suggests that spotted hyena subordinate siblings would be more likely to “accept” the dominance relationship when they are older. By doing so, subordinate siblings would probably incur less of a risk of starvation because the occurrence of facultative siblicide in spotted hyena litters typically occurs before they reach three months of age (Hofer and East 1997, Hofer and East 2008). As siblings become older, they accumulate reserves and thus older subordinate can probably better cope with hunger – particularly so since they start to feed on solid food from the age of 6 months onwards (Hofer and East 1993c).

The dominant sibling uses aggression to train its subordinate littermate to submit during the initial phase of a suckling bout. Because subordinates were increasingly submissive during the course of a suckling bout, this suggests that dominant siblings probably benefited from a “winner effect”. In blue-footed boobies, a species in which winner and loser effects have been demonstrated (Drummond and Canales 1998), winner and loser effects probably occur along two largely separate axes of learning (Valderrábano-Ibarra et al. 2007). In other behavioural contexts, several authors could demonstrate loser effects but little or no winner effect at all (Francis 1983, Schuett 1997, Rutte et al. 2006). Averaging across studies, winners were twice as likely to win a second fight whereas losers were five times more likely to lose (Fawcett and Johnstone 2010). Fawcett and Johnstone (2010) suggest that this may be a consequence of studies focusing mostly on young or socially inexperienced individuals, and that these are precisely the individuals for which they would predict that loser effects are much stronger than winner effects.

Fawcett and Johnstone (2010) recently developed a game-theoretic model to predict the evolution of winner and loser effects in a theoretical population of unfamiliar contestants that varied in their fighting ability (contestants were defined as either “weak” or “strong”) and were unaware of it. Modelling predicted that young individuals, (i.e. those which never fought and were hence unaware of their own strength), should be particularly willing to engage in a contest for a resource because they should seek to obtain information about their own fighting ability. Young and weak individuals were predicted to show the highest intensity of aggression during the first two fights of their life and to increase submissiveness during subsequent fights. When increasing the value of the resource contestants were fighting for, or when reducing mortality risk, weak individuals were willing to sustain more defeats before submitting, but beyond a certain number of contests, they eventually switched to submission (i.e., showed a loser effect).

Although this model focused on contests between pairs of unfamiliar contestants, and not on an ongoing dyadic relationship (as in chapter 2), its predictions may be applicable to young spotted hyena litters. It suggests that young subordinates in spotted hyena litters are assertive at the beginning of suckling bouts because they are (relatively) unaware of their fighting ability. As they become older, they may learn to protect themselves from escalated attacks by showing submission to dominants right from the beginning of a suckling bout (unpublished data).

In a broader context, dominance hierarchies may arise from several (non-mutually exclusive) processes: intrinsic attributes of individuals (e.g. body size or body mass, Arnott and Elwood 2009), the phenomenon termed rank ‘inheritance’ whereby an offspring gains a rank similar to that held by a parent through behavioural support of their parent (Holekamp and Smale 1991, East et al. 2009), social queues, in which social status is determined by the sequence in which individuals join a group (East and Hofer 2001), or trained winner and loser effects (Chase et al. 1994, Dugatkin 1997, Hemelrijk et al. 2008).

Theoretically, stable dominance relationships may emerge from winner and loser effects even in the absence of any pre-existing differences in fighting abilities between individuals (Van Doorn et al. 2003, Hemelrijk et al. 2008). Such a phenomenon is likely to be involved in the emergence of the dominance relationship between spotted hyena siblings, because: (1) in spotted hyena siblings there is no evidence that initial size asymmetries occur at birth (chapter 2, electronic supplementary material 1), and (2) in complex mammalian societies - such as the spotted hyena - dominance is expected to be the result of more subtle traits than relative body size (East and Hofer 2010).

5.1.3 Female spotted hyena siblings may not be the weaker sex

In adult spotted hyena society, females socially dominate males. During infancy, females are more effective at monopolising access to teats and thus may be more likely to survive intense sibling rivalry than males (Hofer and East 1997, James and Hofer 1999). Several lines of evidence (chapter 2) suggest that females have a competitive advantage against males during sibling rivalry: (1) Females were significantly more often dominant in mixed-sex litters than males (based on 182 mixed-sex litters; a similar trend was reported by Smale et al. (1995) and Golla et al. (1999) on smaller data sets of 17 mixed-sex litters and 10 mixed-sex litters, respectively). (2) Suckling time lost by dominant sisters only increased slightly with increasing assertiveness by subordinate brothers (chapter 2, figure 2d). (3) Females appeared to have a competitive advantage as subordinates because subordinate females tended to reverse within-litter dominance order more often than subordinate males in mixed-sex litters. (4) Subordinate females increased the suckling time lost by their dominant siblings, in particular when the dominant was male (chapter 2, figure 2d). (5) In 5 of the 5 twin litters in which the dominant sibling failed to skew maternal milk in its favour and involved at least one female, the subordinate sibling was female (three had a dominant brother, two had a dominant sister and the sixth litter was all-male). Thus no subordinate male raised with a dominant sister managed to skew access to maternal teats in its favour.

Although the reason for the apparent greater competitiveness of female spotted hyena siblings in acquiring dominance status or in efficiently using counter-tactics as a subordinate remains unclear, two non-mutually exclusive explanations could account for it. First, females may have a general competitive advantage against males because of a sex asymmetry in body size or body mass at birth. If females tend to be larger than their brothers at birth, this might provide them with an initial advantage during early contests, enabling females to obtain within-litter dominant status more often and thus the ability to control access to maternal teats. However, there is currently no evidence that females have a higher body mass than males at birth (based on 5 captive litters, Smale et al. 1995), and as mentioned earlier, no evidence that an initial disparity in body size (mass) influences the outcome of sibling rivalry in this species (see discussion in electronic supplementary material 1 in chapter 2). In addition, as previously mentioned, in spotted hyenas dominance status is probably not related to relative body size or mass because, for instance, larger males submit to substantially smaller females, and males submit to cubs and all adults usually submit to cubs which are offspring of high ranking females (personal observation).

Alternatively, the apparent greater competitiveness of female siblings in achieving dominance more often than males may be related to a higher incentive for females to reach this status. Indeed, as dominant siblings grow faster than subordinates (Hofer and East 1997, 2008, chapter 2) and as adult females that benefit from high growth rates during infancy give birth to their litter at a younger age (range: minimum 2.6 years vs. maximum 5.7 years, Hofer and East 2003), dominance pay-offs in terms of future reproduction may be higher for females than for males. Growth rates during infancy have a strong effect on the age at first parturition because an increase in the growth rate by 1 g/day is equivalent to a reduction in the age at first parturition by 15 days. Under this perspective, females would have a higher incentive than males to become the dominant in a mixed-sex litter. In addition, if they are subordinate siblings in a mixed-sex litter, they would have a higher incentive to attempt to reverse the dominance order, and to effectively use counter-tactics during conflicts to reduce their lost suckling time and increase suckling time lost by dominants. My results are consistent with this idea.

5.2 Factors affecting faecal glucocorticoid metabolite concentrations in spotted hyena siblings

5.2.1 Food transit time and inter-individual variability

The research presented in chapter 3 aimed to develop an “in-house”, non-invasive assay for measuring faecal glucocorticoid metabolite (fGCM) concentrations in spotted hyenas. For this purpose a cortisol-3-CMO assay was developed. The ability of the assay to reliably measure fGCM concentrations in spotted hyenas was tested using several procedures. Several lines of evidence demonstrate that this enzyme-immunoassay is a useful indicator of adrenocortical activity in this species: (1) the assay successfully measured increased concentrations of fGCM after adrenocorticotropic hormone (ACTH) challenges in two captive spotted hyenas, and (2) after anaesthesia of another captive spotted hyena. Also, a radiometabolism study was used to characterise fGCM. It showed that the assay mostly detected metabolites with a polarity higher than cortisol and metabolites that eluted between the positions of cortisol and corticosterone. Palme et al. (1996) suggested that the delay in fGCM excretion roughly corresponds to the transit time of food between the duodenum to the rectum. Previously, Goymann et al. (1999) found that fGCM increased within 24 and 50 hours after the onset of a stressor (i.e., an ACTH challenge, translocation, social stress) in all six captive spotted hyenas sampled. In another study using a radio-immunoassay kit to detect corticosterone metabolites in spotted hyena faeces (Dloniak 2004), the glucocorticoid peak delay occurred within 72 hours in 3 out of 4 captive spotted hyenas and after 96 hours in another individual. In our study, the anaesthesia of one captive spotted hyena at Amersfoort Zoo, Netherlands, resulted in a significant increase in fGCM on the fourth day after anaesthesia, roughly 96 hours after treatment.

Together, these empirical data suggests that inter-individual variability and the volume of food consumed influence food transit times and thus glucocorticoid excretion into spotted hyena faeces (range: minimum 16 hours (Goymann et al. 1999, chapter 3); maximum, 96 hours Dloniak 2004, this study). In addition, there is also an elevated inter-individual variability both in pre-treatment and maximum concentrations of fGCM concentrations as demonstrated by this study (pre-treatment concentrations: 2.1 ng/g of faecal matter; maximum concentrations 50.2 ng/g of faecal matter). It is therefore particularly important to use several samples to determine an individual’s pre-treatment level of glucocorticoids, and to keep in mind that there can be substantial inter-individual variation in glucocorticoid concentrations.

5.2.2 Young spotted hyena cubs experience a stressful environment

A predictable environment and the ability to anticipate and avoid the likely occurrence of a stressor are important determinants of physiological stress levels (Sapolsky 2004). Lactating spotted hyenas in the Serengeti National Park had higher fGCM during periods of intense social instability, i.e., when these females were involved in severe fights within the previous 48h, than during periods of social stability. The fission-fusion society of spotted hyenas should allow low-ranking (subordinate) adult females to avoid stressful social interactions with higher-ranking (dominant) adult females, either by avoiding the locations where encounters with dominant females are likely (Hofer and East 2000, East and Hofer 2001), or by leaving these sites when dominant females arrive (Hofer and East 2000, Goymann et al. 2001). Hence, low-ranking (non-lactating females) may reduce the impact of social stressors by avoiding stressful encounters with dominant females. If such is the case, then they may have a relative degree of control over stress caused by unpredictable social interactions. In comparison, lactating females are forced to regularly return to the communal den (the social centre of a clan) to nurse their offspring, and hence it is more difficult for them to avoid stressful social interactions and fights with other females (Goymann et al. 2001).

By contrast, such control over social stressors may be not available to young spotted hyena juveniles (chapter 3). I found that juveniles less than six months of age had approximately 2.7 higher faecal glucocorticoid metabolite concentrations than when they were aged between 6 and 24 months of age (chapter 3, figure 4). The available evidence suggests that the first six months of age of a juvenile spotted hyena are generally more stressful than when they are older. First, the social environment of young (aged between three and six months of age (the youngest animal for which we collected a faecal sample was aged 84 days)) spotted hyena juveniles is likely to be more unpredictable than when they are older: young juveniles have to learn the numerous members that constitute a clan (about 85 individuals during our study period) and to learn whether their mother confers a dominant or submissive status onto them (East et al. 1993). It is probable that during this period of intense learning, juveniles sometimes fail to recognise all clan members and hence cannot predict with accuracy whether they will receive a submissive or an aggressive response, in particular when their mothers are absent (see e.g. Sapolsky 2004, 2005). Moreover, when their mothers are absent on commuting trips and the cubs are still completely dependent on milk, young juveniles left at a communal den face an unpredictable period that may last for several days and during which they are not nursed (Hofer and East 1993c) and thus experience hunger, weight loss and dehydration (Hofer and East 1997). When spotted hyenas are young, they are more likely to experience these potentially stressful effects than when they are older.

Information on glucocorticoid profiles across development in free-ranging animals is essential to assess how natural stressors shape the behaviour of young animals (Mateo 2006). However, the effect of age on the development of the hypothalamic-pituitary adrenal axis and on glucocorticoid levels of free-ranging animals has received remarkably little attention in the fields of behavioural ecology and behavioural endocrinology. Our study (in chapter 3) is the first to provide insight into the age-related dynamics of glucocorticoids in the spotted hyena. Increased glucocorticoid concentrations may trigger important ontogenetic processes such as birth, hatching or fledging, and dispersal (Wada 2008). Moderate increases in these steroid hormones can stimulate behaviours such as begging in birds (Kitaysky et al. 2001) or promote the learning of anti-predatory vigilance behaviours (Mateo 2006) possibly through the mobilisation of energy in the form of glucose (Sapolsky et al. 2000). The concentrations of fGCM of free-ranging juvenile Belding's ground squirrels (*Spermophilus beldingi*) were highest during the first days after emergence from their natal burrows. This peak in fGCM coincided with a period of learning survival strategies (e.g., responding appropriately to alarm calls), anti-predatory behaviours, and the locations of escape burrows (Mateo 2006). In the same species, in captivity, moderately elevated cortisol levels significantly improved associative and spatial learning of juveniles (Mateo 2008). Many experiments showed that catecholamines can promote the consolidation and storage of novel information (Rozendaal 2002). Chronically high levels of glucocorticoids were reported to impair cognition of animals (e.g Mateo 2008, chapter 2). Other studies found a positive effect of moderate increases in glucocorticoids on memory functions (Buchanan and Lovallo 2001, Rozendaal 2002). Hence, moderately elevated concentrations of glucocorticoids in young spotted hyena juveniles (chapter 3) may also facilitate rapid learning of their social environment. From this perspective, glucocorticoids could enhance the ability of young juveniles to memorise clan members and become competent participants in the complex social interactions typical for this species.

5.2.3 Dominant female siblings are more stressed than dominant males

Dominant female spotted hyena cubs in twin litters had approximately four times higher faecal glucocorticoid metabolite concentrations than dominant males (chapter 4, figure 2). As dominant females are more often the dominant member of mixed-sex twin litters and have a better control over access to milk than dominant males (see section above), it may come as a surprise that they are physiologically more stressed than dominant males. This is probably a consequence of the ‘desperado’ behaviours of subordinates raised with dominant sisters (chapter 2, supplementary figure 2 in electronic supplementary material 2). If in mixed-sex litters dominant females are better at controlling access to maternal teats and more likely to commit facultative siblicide than dominant males, akin to dominant females vs. dominant males in same-sex litters (James and Hofer 1999), then subordinate siblings raised with a dominant female are “at risk”. Therefore they are expected to be more assertive than subordinates raised with a dominant brother, in particular when they are hungry. In line with this expectation, fed subordinates were significantly more submissive to their dominant sister than when they were hungry (chapter 2, supplementary figure 2 in electronic supplementary material 2).

The behavioural results obtained in chapter 2 suggest that subordinates with dominant sisters clearly respected the behavioural dominance convention when fed (chapter 2, supplementary figure 2 in electronic supplementary 2, chapter 2). But when hungry, subordinates did not follow this convention and apparently became more assertive than subordinates with dominant males. This significant change in the response of subordinates from highly submissive when fed to more assertive responses when hungry is likely to be stressful and result in elevate fGCM in dominant sisters because (1) the increased assertiveness of subordinates occurred when their hungry dominant sisters are expected to seek to increase their control over access to maternal teats, (2) periods in which hungry subordinates become more assertive are likely to occur repeatedly as prey levels fluctuate, (3) a dominant sister may be unable to predict when their sibling will switch from a highly submissive to an assertive response. The experience of losing social control should dramatically increase glucocorticoids in dominant individuals in many species (discussed in the following section).

5.2.4 The stress of subordinate siblings

Several recent studies discuss whether the level of physiological stress should vary with social status and which social position (dominant vs. subordinate) is most stressful (Abbot et al. 2003, Goymann and Wingfield 2004, Sapolsky 2004, 2005). Traditionally, subordinate members were considered to be more physiologically stressed than dominants and to show more stress-related diseases (von Holst 1998). This perspective was challenged in a review by Creel (2001), who proposed that in many cooperative social species with high reproductive skew, top-ranking animals (dominants) are socially stressed and show higher concentrations of glucocorticoids than subordinates. Nevertheless, the level of reproductive skew in a society is not the only one aspect of a social system, and more general predictors are likely to influence how social status affects glucocorticoid levels in dominants and subordinates (Abbot et al. 2003, Goymann and Wingfield 2004, Sapolsky 2004, 2005).

In this respect, considering the process by which dominance is obtained and maintained should help to understand which social position is experienced as most stressful (Goymann and Wingfield 2004, East and Hofer 2010). In species where high-ranking animals frequently reassert their dominance by using intense aggression (Sapolsky 2005) such as dwarf mangooses (*Helogale parvula*) or African wild dogs (*Lycaon pictus*) and do not enjoy the social support by closely rank-related group members as in spotted hyena females (see East et al.

2009), dominants often have higher physiological stress levels than subordinates (Goymann and Wingfield 2004). In contrast, if dominance is acquired or maintained via low levels of aggression (e.g., threats) and subordinates respond submissively to such intimidation, then chronic elevated glucocorticoid levels may emerge in subordinate members (e.g. olive baboons *(Papio anubis),* Sapolsky 1982, 1990, Sapolsky et al. 1997, rhesus monkey (*Macaca mulatta)* and squirrel monkey (*Saimiri*), mice (*Mus musculus*), rats (*Rattus norvegicus)*; Sapolsky 2005). To summarise, a pattern of chronic elevated glucocorticoid levels in subordinate members may appear if (1) subordinates in a society experience a higher rate of stressors than dominants, and (2) they have insufficient coping mechanisms such as social support, effective avoidance of dominants or appropriate outlets for frustration (see Abbot et al. 2003, Sapolsky 2005).

In adult Serengeti spotted hyenas, levels of fGCM declined with increasing social status in non-lactating females, and social status in itself had no effect on fGCM of lactating adult females (Goymann et al. 2001). Lactating females had higher fGCM levels than non-lactating females, probably because the energetic demands of lactation overshadowed any effect of social status on fGCM (Goymann et al. 2001). Lower-ranking females may experience higher physiological stress levels than higher-ranking females because they have to commute long distances (up to 70km) for a considerable part of the year to obtain food (Hofer and East 2003). Indeed, in the Masai Mara National Park, where low-ranking females do not have to commute to forage, low-ranking females show similar levels of fGCM than high-ranking females. Rates of aggression are typically low among adult spotted hyena males, and in this sex no relationship between social status and glucococorticoid concentrations was found (in the Serengeti: Goymann et al. 2003, in the Masai Mara: Dloniak 2004).

My thesis provides insight into the endocrinology which underlies the relationship between spotted hyena siblings, and my study (in chapter 4) is the first to investigate the link between dominance status and physiological stress in a mammalian twin litter. I found that subordinate siblings had between 1.4 and 1.9 times higher faecal glucocorticoid metabolite concentrations than dominants (using two different analyses; see chapter 4, figure 1). When litters were less than six months of age, subordinate siblings were likely to be confronted with more stressors than their dominant siblings. Indeed, during the first six months of life, (1) aggression rates by dominants are at their highest (Golla et al. 1999), and (2) dominant siblings were able to exclude their subordinate siblings from access to maternal milk for longer periods (chapter 2, supplementary figure 1a in electronic supplementary material 2) than when older, which may result in subordinate siblings consuming less milk (see above). In 9.6% of litters, the subordinate cub dies from enforced starvation when maternal absence intervals are high (Hofer and East 2008). Furthermore, (3) subordinate siblings received approximately 95 % of all within-litter aggression (chapter 2, methods), i.e. only 5% of all assertive or aggressive acts are initiated by subordinates against dominant siblings. Finally, (4) subordinate siblings are intensively trained to submit (chapter 2, figure 4) when young. The establishment of winner and loser effects may involve steroid hormones (Hsu et al. 2006). Fighting experience as a winner or as a loser may lead to specific changes in glucocorticoid and androgens levels (Hsu et al. 2006), and increased concentrations of glucocorticoids are reported in losers in other species (see e.g. Bernstein et al. 1983, Nuňez-de-la-Mora et al. 1996, Hsu et al. 2006).

These differences in glucocorticoid levels may have further consequences for the health status of both siblings. As chronic stress may favour hypertension or immune suppression (Sapolsky 2005, Hofer and East 2012), stressed subordinate siblings may have a higher susceptibility to diseases. Chronically elevated glucocorticoid concentrations increase the risk of cardiovascular disease through hypertension, elevated heart rate (Sapolsky and Share 1994), a pathogenic cholesterol profile and vasoconstriction of damaged coronary arteries. For example, an experimental decrease in social status of captive female cynomolgus monkeys (*Macaca fascicularis*; Shively and Clarkson 1994) led to dramatically worsened coronary artery atherosclerosis and increased blood pressure. Additionally, elevated glucocorticoid levels impair immune functions, potentially increasing the risk of infectious diseases. Losing in captive rats led to persistent immunological alterations and decreased number of immune cells (Stefanski and Engler 1999). Physiologically stressed subordinate males of cynomolgus monkeys showed increased susceptibility to respiratory infection (Cohen et al. 1997), and stressful subordination increased the risk of succumbing to a leukemia-causing virus in mice (Ebbesen et al. 1991).

Consistent with these findings, Serengeti subordinates less than 12 months of age were more likely to be infected with calicivirus than dominants, and this may have directly reduced their longevity (Goller 2011). Such a reduced immuno-competence may be a consequence of the synergistic effects of increased glucocorticoid concentrations, as shown previously (chapter 4, figure 1), and a generally worse nutritional status in subordinate littermates (Hofer and East 1997, 2008, Wahaj and Holekamp 2006, Höner et al. 2010, chapter 2) than in dominants. Because growth rates of subordinate littermates are lower than those of dominants, subordinate siblings in twin litters possibly allocate fewer body resources to immune functions (Goller

2011). Elevated levels of baseline glucocorticoids were also shown to predict mortality in ring-tailed lemurs (*Lemur catta*) (Pride 2005). In conclusion, elevated glucocorticoid levels in young subordinate siblings of spotted hyenas are a consequence of sibling rivalry, and are likely to decrease offspring fitness.

5.3 Conclusions

In this thesis, I explored the behavioural mechanisms and the endocrine responses to asymmetrical sibling rivalry in free-ranging spotted hyena twin litters. I investigated the processes underlying sibling rivalry in a dynamic framework of tactics and counter-tactics used by dominants and subordinates to gain access to a vital resource: maternal milk. For this purpose, I used a well-developed computer software for detailed video analysis and complex statistical methods. Consistent with predictions from theoretical models of sibling rivalry, the identified mechanisms driving the dynamics of sibling contests comprised “desperado” behaviours of hungry subordinate siblings and trained winner and loser effects. However, in contrast to a key assumption of avian models of facultative siblicide, the control exerted by dominant siblings over access to milk was not absolute. My results emphasise the importance of sibling sex during rivalry and provide new insights on the competitiveness of female siblings in mixed-sex litters. For the first time, using a fully validated non-invasive method, I provide in this thesis an investigation of the effects of age, within-litter social status and sex on physiological stress levels of juvenile free-ranging spotted hyena siblings. My results show that intense sibling rivalry is likely to lead to chronic stress in young subordinate siblings, and that the “desperado” behaviours of assertive subordinates are stressful to dominant siblings.

These results provide significant insights on the complexities of intra-family conflicts in mammals. They should contribute to our understanding of sibling rivalry because they reveal new behavioural processes involved in the within-litter dominance relationship of spotted hyena twin litters, and measure for the first time the - potentially costly - endocrine consequences of intense sibling rivalry.

5.4 References

Abbott DH , Keverne EB, Bercovitch FB, Shively CA, Mendoza SP, Saltzman W, Snowdon CT, Ziegler TE, Banjevic M, Garland T Jr, Sapolsky RM (2003) Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Hormones and Behavior 43: 67–82.

Arnott G, Elwood RW (2009) Assessment of fighting ability in animal contests. Animal Behaviour 77: 991–1004.

Bernstein IS, Gordon TP, Rose RM (1983) The interaction of hormones, behavior, and social context in nonhuman primates. In: Svare BB (eds) Hormones and behaviour, pp 535–561. New York, Plenum.

Brosnan SF, de Waal FBM (2003) Monkeys reject unequal pay. Nature 425, 297-299.

Buchanan TW, Lovallo WR (2001) Enhanced memory for emotional material following stress-level cortisol treatment in humans. Psychoneuroendocrinology 26: 307-317.

Burfening PJ (1972) Prenatal and postnatal competition among twin lambs. Animal Production 15, 61–66.

Chase ID, Bartolomeo C, Dugatkin LA (1994) Aggression interactions and inter-contest interval: how long do winners keep winning? Animal Behaviour 48: 393–400.

Cohen S, Line S, Manuck SB, Rabin BS, Heise ER, Kaplan JR (1997) Chronic social stress, social status, and susceptibility to upper respiratory infections in nonhuman primates. Psychosomatic Medicine 59: 213-221.

Creel S (2001) Social dominance and stress hormones. Trends in Ecology and Evolution 16: 491-497.

Dloniak SM (2004) Socioendocrinology of spotted hyenas: patterns of androgen and glucocorticoid excretion in a unique social system. PhD thesis, Michigan State University.

Drea CM, Hawk JE, Glickman SE (1996) Aggression decreases as play emerges in infant spotted hyaenas: preparation for joining the clan. Animal Behaviour 51: 1323–1336.

Drews C (1993) The concept and definition of dominance in animal behaviour. Behaviour 125: 283-313.

Drummond H, Gonzalez E, Osorno JL (1986) Parent-offspring cooperation in the blue-footed booby (*Sula nebouxii*): social roles in infanticidal brood reduction. Behavioural Ecology and Sociobiology 19: 365-373.

Drummond H, Canales C (1998) Dominance between booby nestlings involves winner and loser effects. Animal Behaviour 55: 1669–1676.

Drummond H, Rodríguez C, Vallarino A, Valderrábano C, Rogel G, Tobón E (2003) Desperado siblings: uncontrollably aggressive junior chicks. Behavioral Ecology and Sociobiology 53: 287–296.

Drummond H (2006) Dominance in vertebrate broods and litters. The Quarterly Review of Biology 81: 3-32.

Dugatkin LA (1997) Winner and loser effects and the structure of dominance hierarchies. Behavioral Ecology 8: 583–587.

East ML, Hofer H, Wickler W (1993) The erect penis is a flag of submission in a female-dominated society: greeting ceremonies in Serengeti spotted hyaenas. Behavioral Ecology and Sociobiology 33: 355-370.

East ML, Hofer H (2001) Male spotted hyaenas *Crocuta crocuta* queue for status in social groups dominated by females. Behavioral Ecology 12: 558-568.

East ML, Höner OP, Wachter B, Wilhelm K, Burke T, Hofer H (2009) Maternal effects on offspring social status in spotted hyenas. Behavioral Ecology 20: 478-483.

East ML, Hofer H (2010) Social environments, social tactics and their fitness consequences in complex mammalian societies. In: Szekely T, Moore AJ, Komdeur J (eds) Social Behaviour: genes, ecology, and evolution, pp 360-390. Cambridge University Press, Cambridge.

Ebbesen P, Villadsen J, Villadsen H, Heller K (1991) Effect of subordinance, lack of social hierarchy, and restricted feeding on murine survival and virus leukemia. Experimental Gerontology 26: 479–486.

Fawcett TW, Johnstone RA (2010) Learning your own strength: winner and loser effects should change with age and experience. Proceedings of the Royal Society London B 277: 1427-1434.

Forbes LS (1989) Environmental variability and genotypic conflicts during reproduction in families of ospreys. PhD thesis, Simon Fraser University, Vancouver, Canada.

Francis RC (1983) Experiential effects on agonistic behaviour in the paradise fish, *Macropodus opercularis*. Behaviour 85: 292–313.

Fujioka M (1985) Sibling competition and siblicide in asynchronously hatching broods of the cattle egret *Bubulcus ibis*. Animal Behaviour 33: 1228-1242.

Golla W, Hofer H, East ML (1999) Within-litter sibling aggression in spotted hyaenas: effect of maternal nursing, sex and age. Animal Behaviour 58: 715-726.

Goller KV (2011) Pathogens in free-ranging African carnivores: evolution, diversity and co-infection. PhD thesis, Humboldt Universität zu Berlin.

Goymann W, Möstl E, Van't Hof T, East ML, Hofer H (1999) Non-invasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. General and comparative Endocrinology 114: 340-348.

Goymann W, East ML, Wachter B, Höner O, Möstl E, Van't Hof TJ, Hofer H (2001) Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyenas. Proceedings of the Royal Society of London B 268: 2453-2459.

Goymann W, East ML, Wachter B, Höner OP, Möstl E, Hofer H (2003) Social status does not predict corticosteroid levels in postdispersal male spotted hyenas. Hormones and Behavior 43: 474-479.

Goymann W, Wingfield JC (2004) Allostatic load, social status and stress hormones: the costs of social status matter. Animal Behaviour 67: 591-602.

Grafen A (1987) The logic of divisively asymmetric contests: respect for ownership and the desperado effect. Animal Behaviour 35: 462–467.

Groothuis TGG, Müller W, von Engelhardt N, Carere C, Eising C (2005) Maternal hormones as a tool to adjust offspring phenotype in avian species. Neuroscience and Biobehavioral Reviews 29: 329-352.

Hemelrijk CK, Wantia J, Isler K (2008) Female dominance over males in primates: self-organisation and sexual dimorphism. PLoS ONE 3: e2678.

Hofer H, East ML (1993c) The commuting system of Serengeti spotted hyaenas: how a predator copes with migratory prey. III. Attendance and maternal care. Animal Behaviour 46: 575–589.

Hofer H, East ML (1997) Skewed offspring sex ratios and sex composition of twin litters in Serengeti spotted hyaenas (*Crocuta crocuta*) are a consequence of siblicide. Applied Animal Behaviour Science 51: 307–316.

Hofer H, East ML (2000) Conflict management in female-dominated spotted hyenas. In: Aureli F, de Waal F.B.M. (eds) Natural conflict resolution, pp 232-234. Berkeley, University of California Press.

Hofer H, East ML (2003) Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective. Evolutionary Ecology 17: 315–331.

Hofer H, East ML (2008) Siblicide in Serengeti spotted hyenas: a long-term study of maternal input and cub survival. Behavioural Ecology and Sociobiology 62: 341-351.

Hofer H, East ML (2012) Stress and immunosuppression as factors in the decline and extinction of wildlife populations: the concepts, the evidence and the challenges. In: Aguirre AA, Daszak P, Ostfeld RS (eds) Conservation medicine: applied cases of ecological health, pp 82-107. Oxford University Press, New York.

Holekamp KE, Smale L (1991) Dominance acquisition during mammalian social development: the ‘inheritance’ of maternal rank. American Zoologist 31: 306–317.

von Holst D (1998) The concept of stress and its relevance for animal behavior. Advances in the Study of Behavior 27: 1-109.

Höner OP, Wachter B, Hofer H, Wilhelm K, Thierer D, Burke T, Trillmich F, East ML (2010) The fitness of dispersing spotted hyaena sons is influenced by maternal social status. Nature Communications 1: 60.

Hsu Y, Early L Wolf, LL (2006) Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. Biological Reviews 81: 33-74.

Hudson R, Trillmich F (2008) Sibling competition and cooperation in mammals: challenges, developments and prospects Behavioural Ecology and Sociobiology 62: 299–307.

James WH, Hofer H (1999) A note on sex ratios and sex combinations in Serengeti spotted hyaenas: siblicide and sub-binomial variance. Applied Animal Behaviour Science 65: 153-158.

Kitaysky AS, Wingfield JC, Piatt JF (2001) Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behavioural Ecology 12: 619–625.

Korsten P, Clutton-Brock T, Pilkington JG, Pemberton JM, Kruuk LEB (2009) Sexual conflict in twins: male co-twins reduce fitness of female Soay Sheep. Biology Letters 5: 663-666.

Machmer MM, Ydenberg RC (1998) The relative roles of hunger and size asymmetry in sibling aggression between nestling ospreys, *Pandion haliaetus*. Canadian Journal of Zoology 76: 181-186.

Mateo JM (2006) Developmental and geographic variation in stress hormones in wild Belding's ground squirrels (*Spermophilus beldingi*). Hormones and Behavior 50: 718–725.

Mateo JM (2008) Inverted-U shape relationship between cortisol and learning in ground squirrels, Neurobiology of Learning and Memory 89: 582–590.

Mills M.G.L. (1990) Kalahari hyaenas. The comparative behavioural ecology of two species. Unwin Hyman, London, UK.

Mock DW, Parker GA (1997) The evolution of sibling rivalry. Oxford University Press, Oxford.

Nuñez de la Mora A, Drummond H, Wingfield J (1996) Hormonal correlates of dominance and starvation-induced aggression in chicks of the blue-footed booby. Ethology 102: 748-761.

Palme R, Fischer P, Schildorfer H, Ismail MN (1996) Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Animal Reproduction Science 43: 43-63.

Parker GA, Mock DW, Lamey TC (1989) How selfish should stronger sibs be? The American Naturalist 133: 846–868.

Ploger BJ, Mock DW (1986) Role of sibling aggression in the distribution of food to nestling cattle egrets (*Bubulcus ibis*). Auk 103: 768-776.

Poole AF (1982) Brood reduction in temperate and sub-tropical ospreys. Oecologia 53: 111-119.

Pride RE (2005) High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). Biology Letters 1: 60-63.

Rowell TE (1974) The concept of social dominance. Behavioural Biology 11: 131-154.

Roozendaal B (2002) Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. Neurobiology of Learning and Memory 78: 578–595.

Sapolsky RM (1982) The endocrine stress-response and social status in the wild baboon. Hormones and Behavior 15: 279–292.

Sapolsky RM (1990) Adrenocortical function, social rank and personality among wild baboons. Biological Psychiatry 28: 862–878.

Sapolsky RM, Share LJ (1994) Rank-related differences in cardiovascular function among wild baboons: role of sensitivity to glucocorticoids. American Journal of Primatology 32:261-275.

Sapolsky RM, Alberts S, Altmann J (1997) Hypercortisolism associated with social subordinance or social isolation among wild baboons. Archives of General Psychiatry 54: 1137–1143.

Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21: 55–89.

Sapolsky RM (2004) Social status and health in humans and other animals. Annual Review of Anthropology 33: 393–418.

Sapolsky RM (2005) The influence of social hierarchy on primate health. Science 308: 648-652.

Schuett GW (1997) Body size and agonistic experience affect dominance and mating success in male copperheads. Animal Behaviour 54: 213–224.

Rutte C, Taborsky M, Brinkhof MWG (2006) What sets the odds of winning and losing? Trends in Ecology and Evolution 21: 16-21.

Shively CA, Clarkson TB (1994) Social status and coronary artery atherosclerosis in female monkeys. Arteriosclerosis, Thrombosis, and Vascular Biology 14: 721-726.

Smale L, Holekamp KE, Weldele M, Frank LG, Glickman SE (1995) Competition and cooperation between littermates in the spotted hyaena (*Crocuta crocuta*). Animal Behaviour 50: 671-682.

Stefanski V, Engler H (1999) Social stress, dominance and blood cellular immunity. Journal of Neuroimmunology 94: 144–152.

Tarlow EM, Wikelski M, Anderson D (2001) Hormonal correlates of siblicide in Galápagos Nazca boobies. Hormones and Behaviour 40: 14-20.

Valderrábano-Ibarra C, Brumon I, Drummond H (2007) Development of a linear dominance hierarchy in nestling birds. Animal Behaviour 74: 1705-1714

Van Doorn GS, Hengeveld GM, Weissing FJ (2003) The evolution of social dominance I: Two-player models. Behaviour 140: 1305-1332.

Wachter B, Höner OP, East ML, Golla W, Hofer, H (2002) Low aggression levels and unbiased sex ratios in a prey-rich environment: no evidence of siblicide in Ngorongoro spotted hyenas (*Crocuta crocuta*). Behavioural Ecology and Sociobiology 52: 348–356.

Wada H (2008) Glucocorticoids: Mediators of vertebrate ontogenetic transitions . General and Comparative Endocrinology 156: 441-453.

Wahaj SA, Holekamp KE (2006) Functions of sibling aggression in the spotted hyaena, *Crocuta crocuta*. Animal Behaviour 71: 1401-1409.

Williams TD (1994) Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. Biological Reviews 68: 35–59.