PROXIMATE AND ULTIMATE DRIVERS OF BREEDING-GROUP CHOICE AND REPRODUCTIVE SUCCESS IN MALE SPOTTED HYENAS (*CROCUTA CROCUTA*)

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by

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   Chapter 5

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“Auntie Eve, what is your job about?”

“I study spotted hyenas. I write stories about spotted hyenas.”

“But do you also sometimes write true stories about hyenas?”

“I cannot tell for sure if they are true but I write my stories based on what I understand of hyenas.”

Interview by Colin Davidian (6 ½ years old)

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Zusammenfassung

Bei gruppenlebenden Arten wird die Fitness von Individuen stark durch die Wahl der Fortpflanzungsgruppe und den sozialen Rang in der Hierarchie der Gruppe beeinflusst. Die Ursachen und Folgen von Unterschieden in der Wahl der Fortpflanzungsgruppe und der Abwanderungsneigung sowie die Mechanismen, durch die der soziale Rang den Zugang zu Paarungspartnern und den Fortpflanzungserfolg beeinflusst, sind weitgehend unbekannt. Diese Arbeit hat drei Hauptziele: (i) Ursachen für die Koexistenz von Abwanderung und Ortstreue (Philopatrie) bei Männchen der Tüpfelhyäne (*Crocuta crocuta*), einem großen, gruppenlebenden Raubtier, zu identifizieren; (ii) den Einfluss des sozialen Ranges und sozialer und physiologischer Eigenschaften auf die Investitionen in die Fortpflanzung und den Fortpflanzungserfolg bei Tüpfelhyänen-Männchen zu ermessen; (iii) die Anwendbarkeit von nicht-invasiven Hormonkonzentrationsmessungen zu verbessern. Die Arbeit verbindet Langzeitdaten über das Verhalten, die Physiologie, das Überleben und den Fortpflanzungserfolg von Tüpfelhyänen-Männchen mit demographischen Daten aus allen acht Clans einer freilebenden Hyänenpopulation in Tansania. In Kapitel 3 zeige ich, dass abgewanderte und philopatrische Tüpfelhyänen-Männchen von ähnlicher phänotypischer Qualität sind, die gleichen Prozesse und Regeln anwenden, um eine Fortpflanzungsgruppe auszuwählen und sich darin niederzulassen, und ähnliche Überlebenswahrscheinlichkeiten und Fortpflanzungserfolge haben. Ich zeige auch, dass der soziale Rang den Zugang zu Paarungspartnern und den Fortpflanzungserfolg beeinflusst, dass philopatrische Männchen durch ihren hohen sozialen Rang einen hohen Fortpflanzungserfolg haben, und dass die Vorteile von Ortstreue die Wahrscheinlichkeit der Männchen erhöht, ihre Geburtsgruppe als Fortpflanzungsgruppe auszuwählen. Die Ergebnisse zeigen, dass die Anzahl der Paarungspartner in der Geburts- und anderen Gruppen das Abwanderungsverhalten und die Abwanderungshäufigkeit bestimmt und die Koexistenz von Abwanderung und Ortstreue bei Tüpfelhyänen-Männchen begründet. In Kapitel 4 entwickle ich eine neuartige Methode zur Standardisierung der Messung von Hormonmetabolitenkonzentrationen wenn wesentliche Änderungen in der Messgenauigkeit eines Immunoassays auftreten. Das Verfahren basiert auf der bei jeder Messung wiederholten Analyse einer kleinen Anzahl von Proben und stellt die Vergleichbarkeit der Messungen her. Es ist eine effektive und effiziente Alternative zur oft undurchführbaren wiederholten Analyse aller Proben. In Kapitel 5 zeige ich, dass

Männchen mit einem hohen sozialen Rang mehr Möglichkeiten haben, Dominanz auszuüben und von der Pufferwirkung sozialer Bindungen zu profitieren als Männchen mit einem niedrigeren Rang. Hochrangige Männchen werden dadurch weniger stark durch physiologischen Stress eingeschränkt als tieferrangige Männchen. Dies ermöglicht es hochrangigen Männchen, einschließlich philopatrischen Männchen, mehr Zeit in fitnessfördernde soziale und sexuelle Aktivitäten zu investieren als tiefer rangige Männchen und besonders viel in Weibchen mit hohem Fortpflanzungswert zu investieren. Diese Ergebnisse stehen im Einklang mit den Ergebnissen von Kapitel 3 und können erklären, warum philopatrische Männchen und hochrangige Einwanderer besonders häufig Nachkommen mit Weibchen von hohem Fortpflanzungswert zeugen. Die Arbeit liefert Einblicke in (i) die Rolle der demographischen Variabilität und der Verteilung von Paarungspartnern für die Evolution von Abwanderung und Philopatrie und die Entstehung von Abwanderungsmustern auf Populationsebene, (ii) Merkmale, die Unterschiede in der Konkurrenzfähigkeit und der Investition in Fortpflanzung zwischen Männchen unterschiedlichen sozialen Ranges verursachen, und (iii) die Rolle des sozialen Ranges und sozialer und physiologischer Eigenschaften bei der Entstehung von Unterschieden in der Lebensgeschichte und des Fortpflanzungserfolgs zwischen Männchen bei gruppenlebenden Arten.

Summary

In group-living species, the choice of the breeding group and the social rank in the hierarchy of the group strongly influence an individual's fitness. Yet the causes and consequences of variation in breeding-group choice and dispersal propensity, and the proximate mechanisms through which social rank influences an individual's ability to access mates and reproduce remain poorly understood. This thesis has three main aims: (i) identify the drivers of the coexistence of dispersal and philopatry among males in the spotted hyena (*Crocuta crocuta*), a large, group-living carnivore; (ii) assess whether and how social rank and social and physiological constraints influence reproductive investment and success in male hyenas; (iii) improve the applicability of non-invasive hormone concentration measurements. The thesis combines long-term data on the behaviour, physiology, survival and reproductive success of male spotted hyenas with demographic data from all eight social groups of a free-ranging hyena population in Tanzania. In Chapter 3, I show that philopatric male and dispersers are of similar phenotypic quality, follow the same process and apply the same rules to choose and settle in a breeding group, and have similar survival and reproductive success. I also show that social rank is a strong determinant of male access to mates and reproductive success in spotted hyenas. Philopatric males gain reproductive benefits associated with their high social rank and these benefits increase the propensity of males to choose their natal group as a breeding group. The results demonstrate that the distribution of breeding females across natal and nonnatal groups is an important determinant of dispersal patterns and of the coexistence of philopatry and dispersal in male spotted hyenas. In Chapter 4, I develop a novel method to standardise hormone metabolite concentration measurements when substantial changes in the accuracy of an immunoassay occur. The method is based on repeated assaying of a small number of samples and restores the comparability of the measurements. The method constitutes an effective alternative to the often impracticable re-assaying of complete sample sets. In Chapter 5, I show that males with a high social rank are less constrained by physiological stress than low-ranking males, owing to their greater opportunities to express dominance and enjoy the buffering effect of social bonds. This allows high-ranking males, including philopatric males, to allocate more time than low-ranking males to fitness-enhancing social and sexual activities, and to focus their sexual investment on females of high reproductive value. These results are consistent with the results of Chapter 3 and may explain why philopatric males and high-ranking immigrants are particularly successful at siring offspring with females of high reproductive value. The thesis provides insights into (i) the role of demographic variability and the distribution of breeding partners in driving the evolution of dispersal and in shaping dispersal patterns within populations; (ii) the traits that underpin differences in competitive ability and reproductive performance between males of different social rank; and (iii) the role of social rank and of social and physiological constraints in shaping differences in life history and reproductive success among males in group-living species.

CHAPTER 1

General introduction

Passing on genes to the next generation is a key outcome of evolution by natural selection in living organisms. Yet life histories and reproductive success of organisms vary greatly (Ellis, 1995; Keller and Reeve, 1994). Inter-individual variation in life history and reproductive success has broad implications for the genetic diversity, the adaptability to environmental change and the viability of populations and species (Caplat et al., 2016; Clutton-Brock, 2016). Understanding the causes and consequences of this variation has been of particular interest to studies in socio-biology and behavioural and evolutionary ecology to gain insights into the evolution of sociality, and the diversity of social and breeding systems (Clutton-Brock, 2016; Keller and Reeve, 1994; Kerth, 2008; Markham and Gesquiere, 2017; Port and Cant, 2014).

In group-living species, where multiple males and females form long-lasting spatial aggregations, the fitness of individuals is strongly influenced by the social environment. First, access to local resources such as food, shelter, breeding sites and breeding partners is usually contingent on group membership, that is, the social integration into a social unit through biased investment in social and sexual activities with conspecifics that belong to the same unit (Akinyi et al., 2017; Clutton-Brock, 2016; Foerster et al., 2016). Individuals should thus establish themselves as members of a social group if they intend to breed. Second, the fitness prospects and reproductive success of group members are influenced by the demographic composition, kin structure, and social organisation of the social unit (Alberts, 2012; Clutton-Brock and Huchard, 2013; Kappeler, 2017; Markham and Gesquiere, 2017). For example, the spatial aggregation of breeding females might give males the possibility to monopolise and mate with multiple females (Andersson, 1994; van Noordwijk and van Schaik, 2004). However, the concurrent aggregation of males intensifies competition among males over access to breeding partners and strengthens selection for traits that enhance the ability of males to attract breeding partners or that confer a competitive advantage over other males (Clutton-Brock and Huchard, 2013; Lukas and Clutton-Brock, 2014). In many species, this leads to dominance hierarchies among males and enables high-ranking males to gain a disproportionate share of reproductive success and monopolise the most valuable females of the group (Alberts et al., 2003; Ellis, 1995; Keller and Reeve, 1994; Kutsukake and

Nunn, 2009; Port and Cant, 2014). Long-term reproductive success of males in group-living species is therefore primarily determined by the number of breeding females in the group and the reproductive performance of males in terms of their ability to secure breeding opportunities by attracting females, complying with the mate preferences of females or excluding competitors (Andersson, 1994; East et al., 2003; Höner et al., 2007; Lukas and Clutton-Brock, 2014). How males decide in which group to breed, how their prospects to access mates and reproduce influence their decisions, and how these decisions impact their fitness is still poorly understood.

1.1 Choosing where to breed

The biotic, abiotic and social environments of individuals living in social groups are typically heterogeneous in time and space. As a result, the quality of social groups – defined as the combination of the ecological, social, demographic and genetic characteristics of a group that affect the fitness of group members – can vary substantially (Boulinier et al., 2008; Bowler and Benton, 2005; Danchin et al., 2004; Doligez et al., 2003). Evolutionary theory predicts that individuals should gather information about the quality of potential breeding groups and choose the breeding group – defined as the biased investment in social relationships with and sexual behaviours towards members of a group – that offers the highest breeding prospects (Boulinier et al., 2008; Clobert et al., 2009; Dall et al., 2005). Breeding-group or 'habitat' selection describes such a behavioural process. Individuals are predicted to emigrate from their natal or current group and establish themselves in another group when their fitness prospects in the natal or current group are lower than their prospects elsewhere (Bonte et al., 2012; Bowler and Benton, 2005).

1.1.1 Dispersal as an adaptive movement

Dispersal – as the non-returning movement from the natal or current area (or social group) to another area (or social group) to breed (Clobert et al. 2009; Greenwood, 1980) – is ubiquitous in natural populations and a key component of the life history of individuals, driving a broad range of social, ecological and evolutionary processes (Bonte et al., 2012; Clobert et al., 2009). By moving from one area or social group to another, dispersers influence their own fitness and shape the genetic structure of populations, population dynamics, disease transmission, adaptive potential of populations, and the geographic range of species (Byrne et al., 2018; Caplat et al., 2016; D'Urban Jackson et al., 2017; Ferrière et al., 2000). In group-living species, dispersal patterns – e.g., the proportion of dispersing and non-dispersing individuals of each sex, the distance travelled, and whether dispersal is done solitarily or collectively – further shape the demographic and kin structure of the social groups, and have broad consequences for the social organisation, social competition within and between the sexes, and sexual selection (Clutton-Brock and Lukas, 2012; Kappeler, 2017; Vullioud et al., 2019). Because of its manifold and multiscale implications, dispersal has been a central topic of empirical scientists and theoreticians from various disciplines, including behavioural ecology, conservation biology, and (meta-)population genetics and dynamics (Brom and Massot, 2016; Caplat et al., 2016; Clobert et al., 2009; D'Urban Jackson et al., 2017; Danchin and Cam, 2002; Gilroy and Lockwood, 2012; Shaw and Kokko, 2014; Travis et al., 2012).

Dispersal is generally considered to be an adaptive strategy to reduce the potential costs of kin competition and inbreeding and to adjust to environmental stochasticity (Bowler and Benton, 2005; Johnson and Gaines, 1990). Interestingly, individuals show great variation in their propensity to disperse within species. For example, in group-living mammals, dispersal is usually male-biased: males are more likely than females to disperse (Greenwood, 1980). In addition, the majority of males usually disperse from the group in which they were born while the remaining small proportion of males either never disperse or only do so after a period of philopatry (Bowler and Benton, 2005; Greenwood, 1980; Lawson Handley and Perrin, 2007). Despite advances in our understanding of the evolution of dispersal and the drivers of sex-biased dispersal, the proximate and ultimate causes of the coexistence of dispersers and philopatric individuals within the same sex of a species remain poorly understood (Bonte et al., 2012; Clobert et al., 2009; Clutton-Brock and Lukas, 2012; Gilroy and Lockwood, 2016, 2012).

1.1.2 Dispersal and its underlying behavioural processes

Increasing evidence suggests that a greater understanding of the drivers of dispersal patterns and inter-individual variation in dispersal propensity would be gained by investigating the behavioural processes underpinning dispersal movements (Bonte et al., 2012; Boulinier et al., 2008). In line with this, a mechanistic conceptual framework was developed. It decomposes dispersal into three behavioural stages: (i) emigration, which designates the permanent departure from the natal group, (ii) transience, which designates the movement between groups in the landscape, and (iii) immigration, which designates the settlement in a new group and breeding-group choice (Baker, 1978; Bowler and Benton, 2005; Clobert et al., 2009). By dissociating each stage, the model posits that each stage can be driven by different factors and have specific benefits and costs. It also considers that the costs arising during one stage can influence an individual's decision to carry onto the next stage (Bonte et al., 2012; Bowler and Benton, 2005; Clobert et al., 2009; Travis et al., 2012). For example, gathering information during the transience stage can be costly in terms of increased risk of injury or mortality and missed opportunity to reproduce (Bonte et al., 2012; Danchin and Cam, 2002; Young and Monfort, 2009). Furthermore, access to high-quality groups and mates may be more competed for than access to groups and mates of lower quality (Stamps, 2006; Stamps et al., 2005; van der Jeugd, 2001). The propensity of males to engage in dispersal, their ability to establish themselves and breed in a new group, and the quality of their breeding choice is thus usually predicted to depend on traits that influence their prospection efficiency, competitive ability, and social skills (Bonte et al., 2012; Clobert et al., 2009; Stamps, 2006; van der Jeugd, 2001). This mechanistic conceptual framework has proven useful to explore how the costs associated with each stage of the dispersal movement influence individual dispersal decisions and promote the emergence of phenotypic differences between philopatric individuals and dispersers (that is, "dispersal syndromes"; Bonte et al., 2012; Buoro and Carlson, 2014; Gilroy and Lockwood, 2012; Travis et al., 2012).

1.1.3 Assessing the causes and consequences of dispersal and philopatry

1.1.3.1 Making inferences from observed dispersal patterns

Owing to the difficulty to monitor individuals throughout their lifetime and obtain information on the origin and/or destination of individuals, many studies focus on the emigration movement and make inferences on the adaptive value of dispersal and philopatry from observed dispersal biases. For example, the observations that high proportions of males disperse in group-living mammals was suggested to reflect a selective advantage of dispersal over philopatry (Bowler and Benton, 2005; Frank, 2013; Johnstone, 2000). As a result, in group-living species with a high proportion of dispersers, dispersal was considered to be an adaptive strategy of phenotypically superior males that enhances breeding prospects, whereas male philopatry was either neglected or considered as a “best-of-a-bad” job pursued by phenotypically inferior males (Bowler and Benton, 2005).

Recent studies highlighted that using observed dispersal patterns may lead to erroneous interpretation of the underlying behavioural process(es) because dispersal patterns can emerge from multiple interacting drivers and similar dispersal patterns may emerge for different reasons (Boulinier et al., 2008; Danchin and Cam, 2002; Gilroy and Lockwood, 2012; Serrano et al., 2008; Vardakis et al., 2015). Other studies showed that dispersal may not necessarily reduce kin competition and inbreeding risk, in particular in species living in large groups with promiscuous breeding where the genetic structure of the population may be weak (Lukas et al., 2005; Quirici et al., 2011). Furthermore, gathering information about potential breeding groups may not necessarily incur high costs, as is the case in species where individuals can acquire information without prospecting – for example, by using cues from individuals that immigrated into their group – or when prospection involves short extra-territorial excursions rather than permanent emigration (Cote and Clobert, 2007; Soulsbury et al., 2008). Theoretical work showed that variability in dispersal decisions can emerge in the absence of movement costs and inter-individual phenotypic differences (Gilroy and Lockwood, 2012). Finally, the costs associated with dispersal may not only be incurred during the transience and settlement stages; dispersal may incur short-term reproductive costs after settlement in the new group because of the loss of social bonds and social rank and reduced familiarity with the environment (Danchin and Cam, 2002; Debeffe et al., 2015; Günther et al., 2017). These studies concluded that, to understand the evolution of dispersal and the coexistence of dispersal and philopatry, it is essential to emphasise the process(es) leading to breeding-group choice – that is, integrate the study of dispersal within the concept of habitat selection – and assess the fitness consequences of the choices made by males.

1.1.3.2 Characterising breeding-group choice

Most theoretical and empirical studies that investigate the causes of inter-individual variation in decisions about dispersing focus on the costs and benefits of the movement associated with dispersal. By doing so, they assume that the dispersal movement (the propensity to disperse or the distance travelled) is the trait under selection (Gilroy and Lockwood, 2012; Serrano et al., 2008; Vardakis et al., 2015). The distinction between dispersers and philopatric males is based on whether or not they completed the three-stage process (departure, transience, and settlement) described by the mechanistic framework (Bonte et al., 2012; Bowler and Benton, 2005; Travis et al., 2012). Such binary categorisation implicitly assumes that only (successful) dispersers express a breeding-group choice upon their settlement in a new group; the possibility that philopatry may also be an informed breeding-group choice is rarely considered. In addition, by focusing on the breeding-group choices made by dispersers (that is, the dispersal destination), studies usually exclude the natal group as a potential breeding group (Serrano et al., 2008; Vardakis et al., 2015). By emphasising the movement of males, these studies implicitly assume that philopatric males and dispersers undergo different decision processes and adopt two distinct strategies.

Emphasising male settlement decisions rather than movements may provide a better understanding of the processes leading to the coexistence of philopatry and dispersal (Boulinier et al., 2008; Danchin and Cam, 2002; Gilroy and Lockwood, 2012; Höner et al., 2007). Such an approach requires the identification and characterisation of the potential breeding groups that males can choose among (Boulinier et al., 2008; Gaillard et al., 2010; Selonen et al., 2009; Serrano et al., 2008; Vardakis et al., 2015). Potential breeding groups – that is, groups that are both available and accessible – should thus include both the natal group and other groups, irrespective of whether males end up being classified as philopatric or dispersers. The ability to collect such empirical data may be hampered by (i) an inappropriate scale of the study population – when the number of social groups monitored is not large enough to capture most dispersal movements and to know the origin and destination of dispersers in the population – and (ii) the lack of knowledge of the key determinants of male fitness and the factors that determine the quality of the potential breeding groups (Gaillard et al., 2010).

1.1.3.3 Estimating fitness consequences of choice

Assessing the fitness consequences of dispersal and philopatry requires data on survivorship and reproductive success of a representative number of dispersers and philopatric individuals. Obtaining unbiased fitness estimates for dispersers and philopatric males in free-ranging populations is particularly challenging owing to the usually unknown fate of males that disperse outside the study population (Doligez and Pärt, 2008; Johnson and Gaines, 1990; Nevoux et al., 2013). Although efficient analytical tools have been developed to account for the incomplete monitoring of study animals, the reliability of the inferences derived from these tools still heavily relies on the ability to evaluate the quality of the empirical data (Cayuela et al., 2018; Zimmerman et al., 2007). Besides the issues associated with the size of the study population, another overlooked source of bias in fitness estimates is the method applied to estimate genetic parentage. Because genetic parentage analyses compare the genetic profile of offspring with that of potential fathers, the accuracy and reliability of offspring-father assignments depends heavily on the criteria used to define the pool of potential fathers, in addition to the quality of genetic data (Marshall et al., 1998). An inappropriate criterion for candidate fathers and a low proportion of genetically typed potential fathers can impair the validity of paternity assignments and thus estimates of male reproductive success (Bercovitch, 1986; Höner et al., 2008).

Assessing the temporal variation in survivorship and reproductive success is also fundamental to investigate potential trade-offs that may lead to differences in life history and reproductive tactics between philopatric males and dispersers (Doligez and Pärt, 2008; Germain et al., 2017; Nevoux et al., 2013). The time span of data collection should thus be adjusted to the lifespan of study animals to cover a representative portion of the reproductive career of males after their breeding-group choice.

Interpreting fitness estimates and making inferences on the consequence of philopatry and dispersal may further be hampered by the difficulties to disentangle the effects on fitness of male phenotype, sexual maturity, the quality of the chosen breeding group and the dispersal movement itself (Debeffe et al., 2015; Doligez and Pärt, 2008; Höner et al., 2008; Nevoux et al., 2013; Tarwater and Beissinger, 2012; Vardakis et al., 2015). Empirical studies that addressed all these conceptual and methodological issues in free-ranging group-living animals are currently lacking because of the difficulty to collect detailed information on the ecological, social, demographic and genetic characteristics of social groups, as well as their dynamics, and on the fitness of individuals over a sufficiently large spatial and temporal scale. This is particularly true for studies on long-lived species with slow reproductive rates, such as large mammals (Clutton-Brock and Sheldon, 2010; Johnson and Gaines, 1990).

1.2 Gaining access to mates

The extent to which a male’s breeding-group choice translates into reproductive success largely depends on the male’s performance, that is, his investment in traits and behaviours that increase his chances to secure mating opportunities with females (Careau and Garland Jr, 2012; Moore and Hopkins, 2009; van Noordwijk and van Schaik, 2004). For example, in many species the males who invest in excluding rivals from access to females and/or who comply with mate preferences of females by investing in the establishment of social bonds and friendly relationships with females usually have a greater chance to sire offspring than the males who do not (e.g., Alberts et al., 2003;East et al., 2003; Höner et al. 2007; Setchell et al. 2010).

Intrasexual competition for access to limited resources – the most valuable resource for reproduction being breeding females – is an important process in group-living species. In species where social groups contain numerous adult males, this often leads to the establishment of dominance hierarchies among males. These hierarchies are often established based on the relative competitive ability of males as defined by individual attributes, such as aggressiveness, body size and age, or by social attributes, such as the number of coalitionary partners (Alberts et al., 2003; Clutton-Brock and Huchard, 2013; East and Hofer, 2001; Foerster et al., 2016; Vullioud et al., 2019; but see Chase et al., 2002 and Franz et al., 2015 for the influence of self-reinforcing processes such as winner-loser effects on hierarchy establishment and maintenance). Sexual selection theory predicts that the most dominant and competitive males should monopolise access to most breeding females and/or females of the highest reproductive value, and sire a large proportion of offspring of the group (Alberts et al., 2003; Ellis, 1995; Keller and Reeve, 1994; Kutsukake and Nunn, 2009; Port and Cant, 2014), leading to unequal portioning of reproduction within a group, that is, reproductive skew (Clutton-Brock and Huchard, 2013).

In most group-living species, male reproductive success is influenced by male social rank (Cowlishaw and Dunbar, 1991; Ellis, 1995; Keller and Reeve, 1994). Yet dominant males rarely monopolise reproduction in their social group (Alberts et al., 2003; East et al., 2003). Furthermore, the strength of the association between male social rank and reproductive success can vary greatly within and between species (Alberts et al., 2003; Cowlishaw and Dunbar, 1991; Ellis, 1995; Keller and Reeve, 1994) as a result of female reproductive behaviour, such as mate preferences that are independent of male social rank, polyandry and post-copulatory cryptic mate choice (East et al., 2003; van Noordwijk and van Schaik, 2004; Young et al., 2013), the degree of female oestrus synchrony and gregariousness (Alberts et al., 2003; Engh et al., 2002; Wroblewski et al., 2009) and the use of alternative reproductive tactics by subordinate males (Rubenstein and Nuñez, 2009; Young et al., 2013).

1.2.1 Rank-related reproductive skew

The degree of male reproductive skew strongly influences the genetic structure of the social group and can influence male breeding-group choice and dispersal decisions through complex eco-evolutionary feedbacks (Auld and Rubio de Casas, 2013; Kappeler, 2017; Kokko and Johnstone, 1999; Kutsukake and Nunn, 2009; van Noordwijk and van Schaik, 2004). Investigating the causes and consequences of intrasexual competition, sexual conflicts and reproductive skew is therefore of interest to understand the evolution of group-living and the variability of social and breeding systems observed within and among species (Clutton-Brock and Huchard, 2013; Kappeler et al., 2013; Port and Cant, 2014).

These questions have stimulated the development of theoretical models of reproductive skew that can be categorised into two main groups, each making different assumptions about the underlying mechanisms (Clutton-Brock et al., 2001; Keller and Reeve, 1994). The first group of models are referred to as 'transactional' models and focus on the constraints imposed by the benefits of maintaining the stability of the social group. They posit that dominant males should concede a share of reproduction to subordinates as a staying incentive or that the subordinates should restrain reproductive investment to avoid eviction from the group (Johnstone, 2000; Port and Kappeler, 2010). The second group of models are referred to as 'tug-of-war' or 'limited control' models. They focus on the costs that (selfish) investment in reproductive competition imposes on group productivity, such as reduced female fecundity and reduced offspring survival due to reduced paternal or cooperative care. They posit that males compete over limited group productivity and that dominant males should adjust their investment in conflict and level of aggressiveness in ways that maximise their share of group productivity (Johnstone, 2000; Reeve et al., 1998; Rubenstein and Shen, 2009).

Most of these models are based on the premise that the dominant male has an advantage over subordinates in terms of greater mate attractiveness and/or competitive ability (Alberts et al., 2003; Cant and Reeve, 2002; Port and Cant, 2014; Reeve et al., 1998). They also usually focus on the factors that limit the ability of the dominant male to monopolise reproduction within the social group (Alberts et al., 2003; Port and Kappeler, 2010). Although these models have greatly advanced our understanding of the potential proximate and ultimate causes of variation in reproductive skew within and between species (Alberts et al., 2003; Kutsukake and Nunn, 2009; Port and Kappeler, 2010), we still know little about the nature of rank-related competitive ability and the proximate mechanism through which male social rank influences male access to mates and reproductive success (Careau and Garland Jr, 2012; Cavigelli and Caruso, 2015; Dantzer et al., 2016; Moore and Hopkins, 2009).

1.2.2 Determinants of male competitive ability and performance

Understanding how male social rank influences reproductive success is best done by investigating the influence of social rank on male performance because the traits and behaviours defining male performance are the likely traits under selection (Careau and Garland Jr, 2012; Cavigelli and Caruso, 2015; Moore and Hopkins, 2009).

Comparisons between groups of the same and different species demonstrated that male investment in reproduction and male reproductive success strongly vary with the number of male competitors in the group, and that reproductive skew usually decreases when the number of competitors increases (Alberts, 2012; Alberts et al., 2003; Cowlishaw and Dunbar, 1991; Gogarten and Koenig, 2012; Setchell et al., 2005). These results suggest that the intensity of intrasexual competition plays an important role in shaping rank-related performance (Cowlishaw and Dunbar, 1991; Gogarten and Koenig, 2012). These studies thus suggest that a male’s performance may be primarily determined by its density-dependent ability to cope with the costs of competition. They are inconsistent with the hypothesis that intrinsic physical attributes such as body size or weaponry drive male performance because one prediction from this latter hypothesis should be that the performance and success of a male should be independent of the presence of other, lower-ranking males.

1.2.2.1 Physiological constraints

Physiological processes may be mediators of rank-related performance and reproductive success among males. Intrasexual competition over acquisition and maintenance of social rank and over access to mates can be associated with costly changes in physiological traits such as the concentration of glucocorticoids (Creel et al., 2013; Goymann and Wingfield, 2004), hereafter termed ‘physiological stress’. Glucocorticoids are key physiological mediators of the endocrine control mechanisms that regulate essential biological functions (McEwen and Wingfield, 2003; Romero et al., 2009), and can influence individual life history and fitness (Dantzer et al., 2016; Ricklefs and Wikelski, 2002). While an acute increase in the secretion of glucocorticoids constitutes an adaptive physiological response to a brief ecological or social challenge, maintaining elevated physiological stress as a result of sustained exposure to challenges can disrupt an individual’s endocrine control mechanism, its ability to respond adequately to subsequent challenges and impair its fitness (DuRant et al., 2016; Koolhaas et al., 2011; MacLeod et al., 2018; McEwen and Wingfield, 2003; Romero et al., 2009).

The social rank of a male typically influences his exposure to social challenge and may also influence his ability to cope with the associated physiological demand. By definition, an improvement in social rank increases a male’s likelihood to win agonistic interactions and the number of competitors (that is, subordinate males) he can dominate. High-ranking males also often have more coalition partners and stronger social bonds than low-ranking males (Cavigelli and Caruso, 2015; Creel et al., 2013; Goymann and Wingfield, 2004; Sapolsky, 2005; Sapolsky and Ray, 1989). Empirical studies showed that dominating a social interaction can elicit a weaker stress response and lead to a faster recovery to pre-conflict concentrations than defeat (Koolhaas et al., 2011; Øverli et al., 1999). Furthermore, being able to redirect aggression onto a lower-ranking third party after being defeated and having strong social bonds or coalitionary support may serve as stress coping outlets and a social buffer that enable individuals to downregulate their physiological stress (Abbott et al., 2003; Creel et al., 2013; Sapolsky, 2005; Young et al., 2014). Thus, low-ranking males, which have a relatively low dominance potential, may have less of a buffer to cope with socially-induced stress – that is, experience stronger physiological constraints – (DuRant et al., 2016; Romero et al., 2009). They should therefore be under stronger selective pressure to adjust their behaviour in ways that minimise their exposure to conflicts and allow them to downregulate their physiological stress (Briffa and Sneddon, 2007; Goymann and Wingfield, 2004; Raulo and Dantzer, 2018; Ricklefs and Wikelski, 2002; Romero et al., 2009; Sapolsky, 2005; Stier et al., 2012; Teunissen et al., 2018).

Quantifying glucocorticoid concentrations as a key component of the physiological stress of animals can be done non-invasively using enzyme immunoassays that are specifically designed to measure the concentration of hormone fragments (‘metabolites’) in faecal or urine samples. Measurements of physiological stress from faeces and urine provide an integrated measure of the endocrine activity over a relatively long period of time (that is, from hours to days, depending on excretion frequency and food transit; Goymann, 2005). They reflect the combination of baseline concentrations, endocrine responses to social conflicts (of potentially varying amplitude and frequency), post-conflict recovery (of varying latency), and downregulation by use of coping outlets and social buffer and therefore are a biomarker for “allostatic load” (Goymann, 2005; Goymann and Wingfield, 2004). Although enzyme immunoassays designed for faecal and urine samples are known to have some drawbacks, such as their tendency to fluctuate in the accuracy of their measurements (Noble et al., 2008; Palme, 2019; Wasser et al., 2010; Watson et al., 2013), they are the most appropriate approach to study the social and ecological factors that influence physiological stress in free-ranging populations (Hofer and East, 2012).

A number of socio-endocrine studies compared the physiological stress of high-ranking and low-ranking males during and outside the breeding season with the objective to assess the cost of sexual investment and mate competition for each class of males (Bergman et al., 2005; Corlatti et al., 2012; Creel, 2001; Gesquiere et al., 2011; Girard-Buttoz et al., 2014; Setchell et al., 2010). Most of these studies were conducted in species (primarily cooperatively-breeding species and primates) where only the dominant males invested in reproduction or where the dominant males engaged in costly sexual behaviours, such as mate-guarding, whereas subordinate males adopted alternative reproductive tactics that circumvent direct competition. These studies could not disentangle the effects of male social rank, sexual investment, and exposure to social conflicts on physiological stress. We therefore currently do not know whether and how rank-related dominance potential and physiological constraints mediate variations in male reproductive investment, performance, and ultimately, reproductive success (Beehner and Bergman, 2017; Careau and Garland Jr, 2012; Cavigelli and Caruso, 2015; Dantzer et al., 2016; Moore and Hopkins, 2009; Ricklefs and Wikelski, 2002).

1.2.2.2 Social constraints

Most studies that investigated the causes of reproductive skew focused on the physiological costs of sexual investment and competition over access to mates and how these costs may constrain the ability of males to invest in reproduction (Emery Thompson and Georgiev, 2014; Port and Kappeler, 2010). Although male reproductive investment and performance is likely to be the main trait under selection, selection may also act on male investment in social activities to establish or re-inforce their social rank (Cant et al., 2006; Port and Cant, 2014). Social activities are also essential for the development and maintenance of social bonds and coalitions, and can strongly influence male current and future reproductive success (East and Hofer, 2001; Vullioud et al., 2019; Willisch et al., 2010; Wiszniewski et al., 2012; Young et al., 2013). The possibility that male reproductive investment may not only be influenced by the physiological costs of sexual activities and mate competition but also be constrained by the time allocated to – and the physiological costs of – other fitness-enhancing activities, such as social activities, has not been previously studied (Briffa and Sneddon, 2007; Dunbar et al., 2009; Raulo and Dantzer, 2018).

1.3 Aims and objectives of the thesis

This thesis aims to identify proximate and ultimate drivers of variation in reproductive success among males in a social mammal, the spotted hyena (*Crocuta crocuta*). The thesis focuses on breeding-group choice and social rank, two traits that shape the life history and social environment of group-living animals. It combines long-term data on the behaviour, physiology, survival and reproductive success of males with demographic data from the eight clans composing a free-ranging population of spotted hyenas in the Ngorongoro Crater (Tanzania). The thesis focuses on three main aims:

- **Identify the proximate and ultimate drivers of the coexistence of philopatry and dispersal in males.** To achieve this, I characterise and compare the breeding-group choices of philopatric males and dispersers. I further estimate and compare the fitness of philopatric males and dispersers – with a particular emphasis on the quantitative (number of offspring produced) and qualitative (expected survival and reproductive value of the offspring produced) components of male reproductive success – and assess the factors that influence male reproductive success.

- **Study the proximate mechanism by which male social rank influences reproductive success.** I investigate whether and how male social rank influence male reproductive performance *via* a mechanistic physiological pathway. I specifically tested whether the social correlates of social rank influence the interplay between physiological stress and male social and sexual behaviours. I further investigated the role of social and physiological constraints in shaping rank-related differences in reproductive investment and ultimately, reproductive skew.

- **Improve the applicability of non-invasive assessment of animal physiological state for long-term research projects.** I study the impact of changes in the accuracy of immunoassays on the comparability of hormone metabolite measurements. I develop a method to standardise – that is, restore the comparability of – hormone metabolite measurements when substantial changes in the accuracy of an immunoassay occur. This method constitutes an alternative to the costly, time-consuming, and often impracticable re-assaying of complete sample sets.

The three aims of my thesis were addressed as three chapters:

Chapter 3 – Why do some males choose to breed at home when most other males disperse?

In this chapter, I investigate the proximate and ultimate causes of the coexistence of philopatry and dispersal among male spotted hyenas. I test predictions derived from the two main hypotheses proposed to explain the coexistence of philopatry and dispersal within the same sex of a species. The first hypothesis originates from the dispersal theory and posits that philopatric males and dispersers coexist because high dispersal costs prevent phenotypically inferior males from dispersing and achieving high reproductive success. The second hypothesis is derived from the habitat selection theory and posits that the coexistence of philopatry and dispersal is the result of all males applying the same rules of breeding-group choice and choosing philopatry or dispersal depending on whether the natal group or a nonnatal group offers the highest fitness prospects. I compare the pattern and quality of breeding-group choice made by philopatric males and dispersers and the fitness consequences of their choice, in terms of their survival, age and tenure at first reproduction, and the number and quality of the offspring they sired. I thereby disentangle the influence of breeding-group quality, male phenotype, social rank, and native origin on male reproductive success. I further investigate the influence of the spatial heterogeneity in breeding group quality across natal and nonnatal clans, and of male prospects to acquire a high social rank on the breeding-group choice by individual males and the dispersal patterns at the level of the population.

Chapter 4 – Determining hormone metabolite concentrations when immunoassay accuracy varies over time

In this chapter, I discuss potential problems associated with non-invasive hormone concentration measurements, specifically the effect of variation in the accuracy of immunoassays on the comparability of measurements of faecal hormone metabolites. I present a novel method that standardises measurements of hormone metabolite concentrations, and thereby restores their comparability, based on repeated assaying of a small number of samples. To develop the method, I use data on faecal cortisol metabolite concentrations which were initially measured in several batches with differing accuracy and later re-measured in a single batch with the same accuracy. I adapt statistical concepts and tools by implementing intuitive criteria derived from the field of analytical endocrinology. The standardisation procedure provides explicit tools and guidelines to (i) detect changes in the accuracy of the immunoassay, (ii) identify groups of samples that were assayed with different accuracy and thus need to be re-assayed or standardised, (iii) model the relationship between initial and re-measured cortisol metabolite concentrations and retrieve a standardisation formula, (iv) assess the reliability of the standardisation formula, and (v) estimate the minimum number of samples that need to be re-assayed to produce a reliable standardisation formula to apply to all other samples.

Chapter 5 – Physiological stress as a mediator of rank-related differences in male reproductive investment in a social mammal

In this chapter, I investigate the role of physiological stress as a proximate mediator of rank-related variation in reproductive success. I build on the concept that reproductive performance (e.g., investment in sexual behaviours) is a trait under selection and introduce the novel concept of dominance potential – the integrative measure of the ratio of dominance and coping opportunities to the challenges received by an individual. I test the existence of a mechanistic pathway linking rank-related dominance potential, physiological stress, performance, and reproductive success. I first investigate whether males of different social rank experience different physiological constraints. I specifically test whether low-ranking males experience higher physiological stress than high-ranking males in contexts of social activities and competition over access to mates. Second, I investigate whether rank-related physiological constraints are substantial enough to cause differences in reproductive investment between low-ranking and high-ranking males. I specifically test whether males of different social rank show different behavioural trade-offs between male allocation of time and physiological resource to social integration, reproduction, and self-maintenance. I further test whether these constraints influence male reproductive tactics in terms of the magnitude of their reproductive investment and how they allocate their reproductive effort among females of different reproductive value. Finally, building on the results of Chapter 3, I investigate whether philopatric males and dispersers experience different social and physiological constraints.

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CHAPTER 2

General description of the study population and methods

2.1 The study species: the spotted hyena

Spotted hyenas are large carnivores that live in multimale, multifemale social groups (‘clans’) that contain up to 130 members (Vullioud et al., 2019). Spotted hyena societies are characterized by a stable linear hierarchy (East and Hofer, 2001; Frank, 1986), a fission-fusion system (Smith et al., 2008), and promiscuous breeding with no distinct breeding season (East et al., 2003; Engh et al., 2002; Kruuk 1972). Dominance relationships within and between the sexes are determined by asymmetries in coalitionary support rather than fighting ability (Vullioud et al., 2019). In the hierarchy of the clan, offspring of both sexes obtain the social rank just below their mother through postnatal maternal behavioural support and social learning – a phenomenon known as “maternal rank inheritance” – (East et al., 2009; Holekamp and Smale, 1991). They normally retain this rank until they disperse or die (Höner et al., 2010; Smale et al., 1993).

Dispersal is strongly male-biased; approximately 85% of males leave their natal clan and immigrate into another clan to breed (see Chapter 3), whereas females usually are philopatric (Höner et al., 2005, 2007). As a result, clans typically consist of females with their offspring, immigrant males and a few reproductively active native (“philopatric”) males. Dispersal disrupts the social bonds and coalition network and reduces the number of supporters (Vullioud et al., 2019). As a result, all native individuals dominate all immigrant males and immigrant males queue for social status (East and Hofer, 2001; Vullioud et al., 2019).

Male-biased dispersal is mainly driven by female mate-choice preferences that evolved to avoid inbreeding (Höner et al., 2007). Breeding-group choice by males and male long-term reproductive success are primarily driven by the number of young females in a clan (i.e., male compliance to female mate preferences; see Chapter 3), rather than by the number of adult male members (i.e., intrasexual competition for mates), the number of unrelated females (i.e., direct inbreeding avoidance by males), and the prey density within a clan territory (i.e., competition for food; Höner et al., 2007, 2010).

2.2 The study population

This doctoral study was conducted based on the behavioural, demographic, physiological, and genetic monitoring of all eight clans of spotted hyenas inhabiting the 250-km² floor of the Ngorongoro Crater. The Ngorongoro Crater is a natural caldera located in the north of Tanzania (3°11'S, 35°34'E) adjoining the eastern border of the Serengeti National Park. It is part of the Ngorongoro Conservation Area, a multiple-use protected area created in 1959. It is ecologically well connected to adjacent habitats and animal populations, with individuals of many species, including the spotted hyena (Albert, 2002), migrating between the different populations.

Since the beginning of the long-term study on spotted hyenas in the Ngorongoro Crater in 1996, between 194 and 580 spotted hyenas inhabited the Crater floor. Clans contained between 3 and 130 individuals who defended access to food within their territory. Empirical evidence showed that the number of members in a clan does not depend on the abundance of prey within the clan territory but on overall prey abundance on the Crater floor (Höner et al., 2005). The sizes of hyena territories in the Ngorongoro Crater (between 15 km² and 40 km²) are smaller than those of clans in the Serengeti National Park in Tanzania (55 km²; Hofer and East 1993) and the Masai Mara in Kenya (62 km²; Holekamp and Dloniak, 2010). Clan territories overlap with territories of neighbouring clans (Figure 1); they also change in size and shift over time as a function of the number of clan members (Höner et al., 2005; Kruuk, 1972) and the intensity with which clan members use particular areas within their territory (Kolowski and Holekamp, 2009; Trinkel et al., 2004).

The study population was highly suitable for the aims of my thesis. The number of clans was sufficient to capture most male breeding-group choices (see section 2.4). The continuous and detailed long-term monitoring of all eight clans of the population (see sections 2.3 and 2.5) allowed me to determine for all philopatric males and dispersers (i) their clan of birth, (ii) the demographic and social characteristics of the potential breeding clans, (iii) the chosen breeding clan, and (iv) their social status (see Chapter 3 and Chapter 5). Access to a large number of habituated individuals also allowed me to closely monitor the behaviour and collect samples of many different males and relate their behaviour to physiological markers (see section 2.6 and Chapter 5). The collection of samples from a large number of offspring and genetic information from almost all reproductively active males throughout the 23-year study period further allowed me to determine the fathers of a large number of offspring with very high accuracy (see section 2.5) and estimate the fitness consequences of their choices (see Chapter 3).

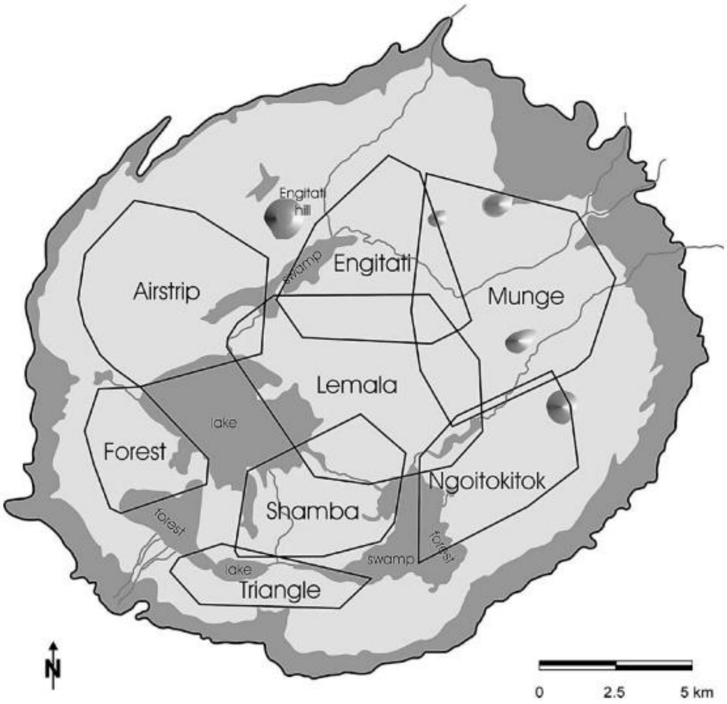

Figure 1: Distribution of spotted hyena clan territories in the Ngorongoro Crater between 1996 and 2015. (Credit: Oliver Höner, used with permission of the author)

2.3 Behavioural and demographic monitoring

Data on the behaviour and life history of the hyenas in the Ngorongoro Crater were collected by me between 2010 and 2013 and by colleagues from the Leibniz Institute for Zoo and Wildlife Research (Berlin, Germany) between 1996 and 2015 as part of an ongoing long-term research project. Spotted hyenas were identified by their unique spot pattern and other natural marks such as ear notches (Figure 2). Individuals were observed during dawn, daytime and dusk (between 0600 and 1900 h) at resting places, communal dens and birth dens, and when encountering them incidentally in other areas of the territory. Their age – and corresponding birth date – was estimated from pelage characteristics, body size, locomotory abilities, behavioural development, and the position, shape and size of the ears of cubs (East et al., 1989; Kruuk, 1972; Matthews, 1939; Pournelle, 1965). Mother-cub affiliations were primarily assigned based on observations of suckling interactions (East et al., 2009).

Figure 2: Example of the diversity in spot pattern and ear notches among individual spotted hyenas. (Credit: Oliver Höner, used with permission of the author)

2.4 Assessment of male breeding-group choice

Breeding-clan selection was defined as the behavioural process that results in a biased investment (a choice) by a male in social relationships and sexual behaviour, which may influence the male’s survival and reproduction (Clobert et al., 2009). A male was defined to choose a breeding clan if he expressed sexual behaviour towards females and invested in joining the social hierarchy of sexually active males in the natal clan (“philopatric” male) or another clan (“disperser” or “immigrant”), respectively, for at least 3 months (Höner et al., 2007, 2010). The date of clan choice was defined as the date of first observation of such behaviour for philopatric males and of first sighting in the new clan territory for dispersers, and defined as the start of a male’s (reproductive) tenure. Males who did not show any sign of sexual activity or had not met the 3-month criterion before their date of last sighting or the end of the study period were excluded from the analysis. Applying these criteria instead of the commonly used age-based categorisation of males – i.e., assigning a dispersal status to males upon attainment of an assumed age of reproductive maturity – avoids introducing methodological biases in the assessment of the behaviour, reproductive success and physiology of philopatric males and dispersers arising from differences in sexual motivation and maturity (Akinyi et al., 2017; Bercovitch, 1997; Höner et al., 2008; see Chapter 3 and Chapter 5).

During the study period, 306 males born in one of the eight study clans chose to breed in their natal clan or one of the other study clans. This corresponds to more than 80% of all study clan males who reached the mean age at clan choice of 3.4 years (see Chapter 3 and Chapter 5).

2.5 Paternity assignment and estimation of male reproductive success

The reproductive success of males was estimated on the basis of large-scale genetic parentage analyses. Tissue, hair, and faecal epithelium were collected from a total of 1246 hyenas using minimally invasive methods and stored in ethanol or dimethyl sulfoxide salt solution until DNA extraction (Höner et al., 2007; Wilhelm et al., 2003). Fluorescent primers were used to amplify nine microsatellite loci with a mean number of alleles per locus of 11.9 (range = 7 to 16). The error rate was low (0.44%) and the total exclusionary power was high (0.999).

Paternities were assigned using maximum likelihood methods as implemented in CERVUS 3.0 (Kalinowski et al., 2007). All philopatric males and dispersers who were clan members at conception – i.e., 110 days before the estimated birth date (Matthews, 1939) – were considered as candidate fathers (see Chapter 3). For 1048 offspring (95.2% of sampled offspring), all candidate males were genetically typed; the mean proportion of typed candidate males was 0.99. Paternities were assigned to 97.2% of the 1101 sampled offspring with 95% confidence.

2.6 Quantification of physiological stress

Male level of glucocorticoids (i.e., physiological stress) was quantified using faecal samples collected immediately after defaecation, thereby ensuring the reliable determination of male identity. Such a non-invasive sampling method is particularly useful to monitor the natural adrenocortical activity of free-ranging animals because it does not involve potentially stressful procedures (e.g., manipulation and immobilisation of individuals) which may not only cause harm to the study animals but also confound measurements (Hofer and East, 2012).

Quantifying physiological stress from animal faeces however requires different analytical methods than quantifying physiological stress from plasma samples (Palme, 2019). This is because the native glucocorticoid hormone of interest that is originally secreted and released into the bloodstream is usually absent – or present only as traces – in faeces. The molecules that are excreted in faeces are hormone metabolites, products of the downstream metabolisation of the native hormone (Goymann, 2005; Palme, 2019). Whereas the chemical structure of native hormones is usually known, and hormone-specific antibodies can easily be developed to directly measure hormone concentrations in plasma, the structure of various hormone metabolites are often not well known unless validation experiments have focussed on precisely identifying the metabolites. The common approach is to apply indirect analytical methods that apply an antibody specific to a known region of the native hormone and that will cross-react with the hormone metabolites that possess the same target region (Wild, 2013).

The analytical method used here is a competitive Enzyme-Linked Immuno-Sorbent Assay (cELISA) using an antibody specific to cortisol (cortisol-3-CMO). Cortisol is a prominent physiological mediator of the endocrine stress response in mammals and is the main circulating glucocorticoid hormone in spotted hyenas (Goymann et al., 2003; Romero, 2004); quantifying cortisol metabolites in faeces thus is appropriate to assess male physiological stress in spotted hyenas. The cortisol-3-CMO immuno-assay was previously developed for spotted hyenas and thoroughly validated physiologically, analytically and biologically; it demonstrated a high affinity of the antibody with cortisol metabolites, the ability of the antibody to measure natural fluctuation in metabolite concentrations and a high precision of measurement (Benhaiem et al., 2012).

The cELISA was conducted using microtitre plates coated with a polyclonal antibody raised in rabbits against cortisol-3-CMO. Faecal extracts containing cortisol metabolites are injected into the wells of the microtitre plate together with known amounts of cortisol conjugated with a peroxidase enzyme (‘tracer’). During the incubation period, the cortisol metabolites from samples and the tracer will compete for the binding sites of the cortisol-3-CMO antibody. After the incubation period, a reagent is added into the wells to activate the peroxidase enzymes from the fraction of the tracer that bound to the antibody. The activated, bound tracer then generates a photometric response (yellow colour) with a colour intensity (expressed as absorbance or optical density) proportional to the amount of bound tracer; it thus inversely proportional to the amount of bound cortisol metabolites. The cortisol metabolite concentration in a sample is then quantified by relating the measured optical density to a calibrated dose–response curve generated by standards of known concentrations in native cortisol (see Chapter 4).

2.7 References

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CHAPTER 3

Why do some males choose to breed at home when most other males disperse?

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Detailed contributions:

The original idea to compare the reproductive success of immigrant and philopatric male spotted hyenas was developed by Oliver Höner (OH). The idea to embed the study within the broader conceptual framework of habitat selection by investigating the distribution of breeding partners across natal and nonnatal groups and its influence on male dispersal propensity and breeding-group choice was implemented by Eve Davidian (ED). Demographic monitoring and collection of behavioral data and genetic material for paternity assessment were conducted by OH, Bettina Wachter (BW), and ED. Laboratory analyses of microsatellites for paternity were conducted by technical assistants (Kerstin Wilhelm and Dagmar Thierer) under the supervision of OH. Assignment of paternities was conducted by OH. Statistical analyses were conceptualized and conducted by ED and Alexandre Courtiol (AC), under the guidance of OH and Heribert Hofer (HH). The manuscript was primarily written by ED and OH, and edited by AC, HH and BW.

MATING BEHAVIOR

Why do some males choose to breed at home when most other males disperse?

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Dispersal is a key driver of ecological and evolutionary processes. Despite substantial efforts to explain the evolution of dispersal, we still do not fully understand why individuals of the same sex of a species vary in their propensity to disperse. The dominant hypothesis emphasizes movements and assumes that leaving home (dispersal) and staying at home (philopatry) are two alternative strategies providing different fitness. It suggests that only individuals of high phenotypic quality can pursue the most beneficial strategy; the others are left to do a "best-of-a-bad" job. An alternative hypothesis emphasizes settlement decisions and suggests that all individuals pursue a single strategy of choosing the breeding habitat or group with the highest fitness prospects; choosing the natal group (philopatry) and choosing a nonnatal group (dispersal) are then outcomes of these decisions. We tested both hypotheses using a long-term study of a free-ranging population of a group-living carnivore, the spotted hyena. We combined demographic data with data on dispersal-relevant phenotypic traits, breeding-group choice, survival, and reproductive success of 254 males. Our results contradict the best-of-a-bad-job hypothesis: philopatric males and dispersers were of similar phenotypic quality, had similar fitness, and applied similar settlement rules based on the fitness prospects in groups. Our findings demonstrate that the distribution of breeding partners can be more important in shaping dispersal patterns than the costs associated with the dispersal movement. The study provides novel insights into the processes leading to the coexistence of philopatry and dispersal within the same sex of a species.

INTRODUCTION

Dispersal is one of the most important yet least understood drivers of ecological and evolutionary processes (*1*, *2*). In almost all sexually reproducing species, some individuals disperse from the habitat or group in which they were born and attempt to breed elsewhere, whereas others either never disperse or only do so after a period of philopatry. Dispersal patterns in terms of the proportion of dispersing individuals or the distances traveled, however, vary greatly between species, populations, and sexes (*3*–*5*). Previous theoretical and empirical studies have advanced our understanding of the evolution of dispersal and the drivers of sex-biased dispersal (*2*, *5*–*8*). Yet, we currently know little about the evolutionary processes leading to the coexistence of dispersers and philopatric individuals within the same sex of a species (*8*–*10*).

The evolutionary theory predicts that individuals should assess the quality of potential breeding habitats and disperse if the fitness prospects in the natal or current habitat are lower than those elsewhere, after accounting for the costs of between-habitat movements (*8*, *11*, *12*). Dispersal may generally confer fitness advantages by reducing potential costs of kin competition and inbreeding or by leaving a deteriorating habitat, whereas philopatry may confer advantages through familiarity with the natal territory and conspecifics, as well as kin cooperation (*2*, *4*, *7*). The dominant hypothesis to explain the coexistence of philopatry and dispersal suggests that, for a given sex of a species, one strategy provides higher fitness than the other but that only individuals of high phenotypic quality can pursue this strategy and maximize their fitness (*4*, *10*, *13*). In line with this hypothesis, the high proportion of male dispersal observed in many group-living mammals (*3*) was suggested to reflect a selective advantage of dispersal (*4*). Because gathering information about breeding groups ["prospecting" (*14*)] and settling in a group are usually considered costly (*8*, *15*), only males of high searching efficiency, high competitive ability, or high social skills were suggested to succeed in settling and securing a breeding position in a new group (*10*, *13*, *16*, *17*). As a result, male philopatry in group-living species with a high proportion of dispersers has been either neglected or considered as a "best-of-a-bad" job pursued by phenotypically inferior males (*4*).

This hypothesis emerged from studies that focused on the costs and benefits of the movement associated with dispersal (*4*, *8*, *18*). It assumes that moving away from or staying in the natal environment is a trait that falls under natural selection and that philopatric individuals and dispersers experience different selection pressures and breed in environments of different quality. Although these assumptions may be valid for some species, the costs and benefits of the dispersal movement may only play a minor role in shaping dispersal patterns in many others (*19*). For example, in species with large social groups and moderate reproductive skew within sexes, the genetic structure of the population may be weak, and leaving the natal group may not necessarily reduce the potential costs of kin competition and inbreeding (*20*–*22*). Males may also prospect and disperse at low search and survival costs if gathering information does not necessitate emigration from the natal group and dispersal distances are short (*23*, *24*). In such systems, males that eventually become philopatric may also prospect, follow the same process of breeding-group selection, apply similar settlement rules, and have the same fitness prospects as dispersers. This has rarely been considered by theoretical studies within the dispersal context (*19*).

To our knowledge, no study has previously investigated whether the coexistence of philopatric males and dispersers (i) results from high dispersal costs preventing phenotypically inferior males from dispersing or (ii) is a consequence of all males applying the same rules of breeding-group choice and choosing philopatry or dispersal depending on whether the natal group or a nonnatal group offers the highest

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fitness prospects. We tested predictions derived from these two hypotheses on a free-ranging population of a group-living mammal, the spotted hyena (*Crocuta crocuta*), for which we previously identified female mate choice as the main driver of male-biased dispersal (*25*). We combined long-term demographic data from the entire population of the Ngorongoro Crater in Tanzania, comprising eight social groups ("clans"), with data on breeding-group choice, dispersal-relevant phenotypic traits, survival, and reproductive success for 41 philopatric males and 213 dispersers. This comprehensive approach provided the rare opportunity to assess the causes and fitness consequences of male philopatry and dispersal in a social mammal in a population still subjected to the processes of natural and sexual selection.

First, we tested whether philopatric males and dispersers differed in their phenotypic quality before clan choice. In spotted hyenas, males are not evicted from their natal clan by other clan members (*26*). Before choosing a clan for breeding, both philopatric males and dispersers undertake excursions to other clan territories and may thereby assess their fitness prospects in potential destination clans (*25*). During these excursions, males may experience aggression from resident males (*27*). A male's prospecting behavior, his chances to settle in a new clan, and the period of time required until clan choice may therefore be influenced by his searching efficiency, his ability to overcome social challenges, and whether he grew up in the chosen clan (*16*, *28*). Because, in spotted hyenas, sons of high-ranking females grow faster, start reproducing earlier, and are more likely to disperse to clans offering higher fitness prospects than lower-born males (*29*), they may be of higher phenotypic quality with respect to clan choice (*16*). Thus, if prospection and settlement incur high costs and these costs prevent phenotypically inferior males from dispersing, philopatric males should comprise a higher proportion of low-born males.

Second, we tested whether philopatric males and dispersers differed in their fitness in terms of the number and quality of offspring sired, age at first reproduction, and survival. Spotted hyenas live in multimale, multifemale social groups, structured by strict linear dominance hierarchies, in which females and their offspring are dominant over immigrant males (*30*, *31*). A male's chance to be chosen as a sire mainly depends on his investment in developing friendly relationships with females rather than his body size or fighting ability (*32*, *33*). Reproduction within a clan is not monopolized by high-ranking individuals, and females and males breed promiscuously (*33*). Male social rank may nonetheless influence male reproductive success because high-ranking males have privileged access to food and females, and may afford to spend more time and energy consorting and developing relationships with females compared to lower-ranking males. Dispersal and philopatry have different implications for the social rank of males in hyenas; dispersers join the new clan at the bottom of the male social hierarchy and only increase in rank with increasing tenure in the clan, whereas philopatric males dominate all immigrant males (*30*, *34*, and this study). Male reproductive success may be additionally influenced by male origin because males that grew up in the chosen breeding clan ("native males") are more familiar with the females and have better knowledge about their quality and preferred whereabouts than males that grew up in another clan ("foreigners"). If male social rank and origin do influence male reproductive success, philopatric males should sire their first offspring earlier and sire more offspring than dispersers. Similar to top-ranking, long-tenured dispersers (*30*), philopatric males may mainly invest in consorting high-ranking females and sire more offspring of high reproductive value (*35*) compared to dispersers. Higher social rank and the associated preferential access to food within the clan territory may additionally cause philopatric males to be in better physical condition and survive better than dispersers.

Third, we investigated whether philopatric males and dispersers differed in the rules they apply when choosing their breeding clan. Female hyenas have complete control over mating (*36*) and apply mate-choice rules to avoid incest; they prefer sires that were born into or immigrated into their clan after they were born (*25*), and females older than 5 years additionally prefer males with long tenures (*33*). The number of females that comply with these rules on the date a male chooses a clan defines the pool of females most likely to breed with a male, that is, clan quality. Previously, males were shown to prefer high-quality clans (*25*). In our system with fluctuating numbers of females in clans, a male's natal clan may occasionally contain the highest number of likely breeding partners. We therefore tested whether, as predicted by habitat selection models, the quality of male clan choice and the propensity to choose philopatry resulted from the combination of (i) male preference for high-quality clans, (ii) the relative availability of high-quality clans among natal clans, and (iii) the predicted benefits associated with philopatry.

RESULTS

Phenotypic quality of males and age at clan choice

The proportion of philopatric sons of low-ranking (0.21, $n = 8$), medium-ranking (0.44, $n = 17$), and high-ranking (0.36, $n = 14$) females did not differ from the proportion of dispersers that were sons of low-ranking (0.19, $n = 36$), medium-ranking (0.30, $n = 59$), and high-ranking (0.51, $n = 99$) females [Pearson's χ^2 test; $\chi^2 = 4.2$, degree of freedom (df) = 2, $P = 0.12$]. The age at which philopatric males chose their first breeding clan (3.3 ± 0.9 years, $n = 40$ males with known birth date) was similar to that of dispersers (3.5 ± 0.7 years, $n = 177$; $U = 3130.5$, $P = 0.25$). The age at clan choice was not influenced by male origin [ordinary least-squares linear model (LM), likelihood ratio (LR) = 0.07, df = 1, $P = 0.79$] or by maternal social rank (LR = 2.48, df = 1, $P = 0.12$) when we controlled for the identities of the natal clan (LR = 17.75, df = 7, $P = 0.013$) and the chosen clan (LR = 11.86, df = 7, $P = 0.11$; whole model, LR = 1.43, df = 16, $P = 0.009$, $n = 214$ males with known birth date and maternal social rank; see table S1).

Male social rank

Dispersers started their reproductive career in the new clan at the bottom of the male social hierarchy and increased in rank with increasing tenure (Fig. 1). In contrast, philopatric males occupied a high social rank in the male hierarchy from the date of (primary) clan choice until they dispersed (secondary clan choice) or died (Fig. 1).

Tenure and age at first reproduction

Philopatric males sired their first offspring after a shorter tenure (1.1 ± 1.3 years, $n = 25$) than dispersers (1.9 ± 1.5 years, $n = 128$; $U = 994.5$, $P = 0.003$). Philopatric males also sired their first offspring at a younger age (4.2 ± 1.4 years, $n = 24$ males with known birth date) than dispersers (5.3 ± 1.6 years, $n = 100$; $U = 643$, $P < 0.001$). Tenure at first reproduction was significantly shorter for males that grew up in the chosen clan than males that grew up in a different clan (LM, LR = 10.41, df = 1, $P = 0.001$) when controlling for maternal social rank

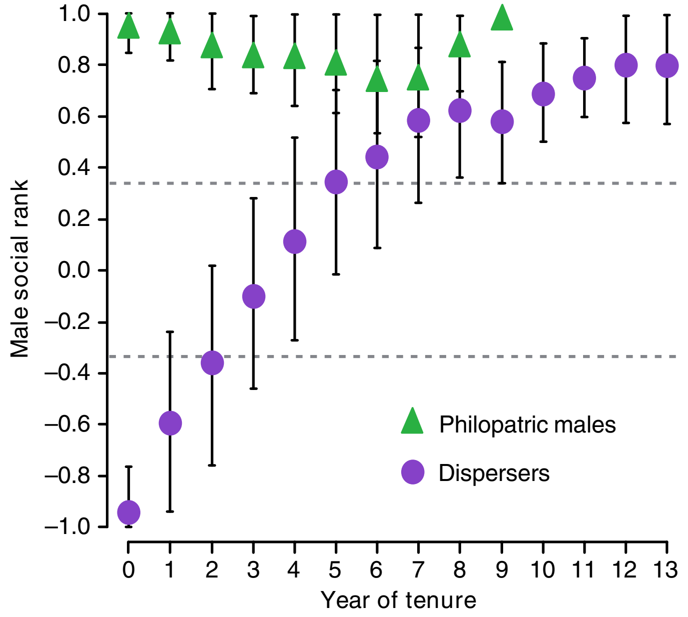

Fig. 1. Change in social rank of philopatric males and dispersers with time spent in the clan (tenure). Data are mean standardized social ranks (symbols) ± SDs (vertical bars). Standardized ranks were calculated at the start of each year of tenure by distributing ranks evenly between the highest (standardized rank +1) and lowest (standardized rank −1) rank in the hierarchy of sexually active males of a clan. Males with standardized ranks within the top, middle, and lower thirds of the total range (delimited by dashed horizontal lines) were high-ranking, medium-ranking, and low-ranking, respectively.

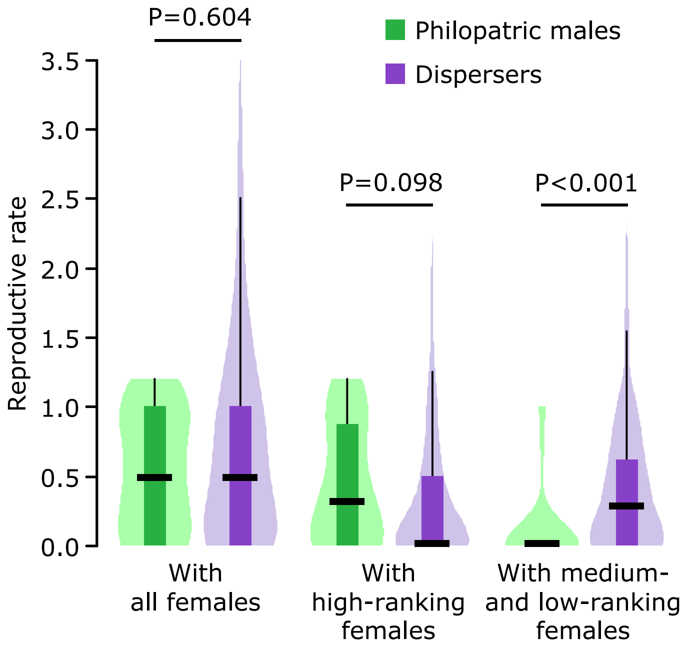

Fig. 2. Comparison of the reproductive rate of philopatric males and dispersers. Reproductive rate is the mean annual number of offspring sired during the first 6 years of tenure. Boxes indicate the interquartile range around the median (horizontal bar), vertical bars represent reproductive rates that lie within 1.5 times the interquartile range, and shaded areas represent the distribution (kernel density estimate) of the data.

(LR = 19.47, df = 1, $P < 0.001$), the number of likely female breeding partners at clan choice (LR = 0.59, df = 1, $P = 0.44$), and the identity of the chosen clan (LR = 15.32, df = 7, $P = 0.032$; whole model, LR = 73.33, df = 10, $P < 0.001$, $n = 136$ males with known maternal social rank; see table S2).

Reproductive success

The mean annual reproductive rate of philopatric males with females of all social ranks (0.53 ± 0.43 offspring per year, $n = 28$ males with tenure ≥1 year) was similar to that of dispersers (0.69 ± 0.73, $n = 168$; $U = 2209.5$, $P = 0.60$; Fig. 2). Philopatric males tended to sire more offspring per year with high-ranking females (0.43 ± 0.44) than did dispersers (0.30 ± 0.47; $U = 2781.5$, $P = 0.098$), and they sired significantly fewer offspring with medium- and low-ranking females (0.10 ± 0.28) than did dispersers (0.39 ± 0.45; $U = 1284$, $P < 0.001$). Philopatric males sired 83% and dispersers sired 39% of their offspring with high-ranking females.

Influence of male origin and social rank on reproductive success

The annual reproductive rate of males native to the chosen clan was lower than that of foreigners [generalized linear mixed model (GLMM), combined effect of male origin and interaction between male origin and year of tenure: LR = 18.95, $P = 0.003$] and increased as male social rank increased (LR = 14.29, $P = 0.003$) when controlling for maternal social rank (LR = 3.62, $P = 0.14$) and the number of likely breeding partners at clan choice (LR = 0.03, $P = 0.99$; whole model, LR = 71.52, $P = 0.001$, $n = 181$ males with known maternal social rank and tenure ≥1 year; see table S3). The annual reproductive rate with high-ranking females was not influenced by male origin (GLMM, combined effect of male origin and interaction with year of tenure: LR = 9.34, $P = 0.16$) and increased as male social rank increased (LR = 15.82, $P < 0.001$) when controlling for maternal social rank (LR = 1.46, $P = 0.41$) and the number of likely breeding partners at clan choice (LR < 0.01, $P = 0.99$; whole model, LR = 56.97, $P < 0.001$, $n = 181$; Fig. 3, A and B; see table S4). The annual reproductive rate with medium- and low-ranking females was lower for native males than foreigners (GLMM, combined effect of male origin and interaction with year of tenure: LR = 39.48, $P < 0.001$) and was not influenced by male social rank (LR = 3.24, $P = 0.12$) when controlling for maternal social rank (LR = 2.99, $P = 0.12$) and the number of likely breeding partners at clan choice (LR = 0.08, $P = 0.89$; whole model, LR = 45.89, $P < 0.001$, $n = 181$; Fig. 3, C and D; see table S5).

Male survival

The survivorship after the date of first clan choice of philopatric males (median from Cox proportional hazards model = 7 years, $n = 41$ males with tenure ≥1 year) and dispersers was similar [median = 8 years, $n = 210$; hazard ratio = 1.4, 95% confidence interval (CI) = 0.79 to 2.46, LR = 1.22, df = 1, $P = 0.27$].

Quality of clans and male clan choice

To test whether the quality of natal clans differed from that of nonnatal clans and whether this affected male clan choice, we ranked the eight study clans according to the number of likely female breeding

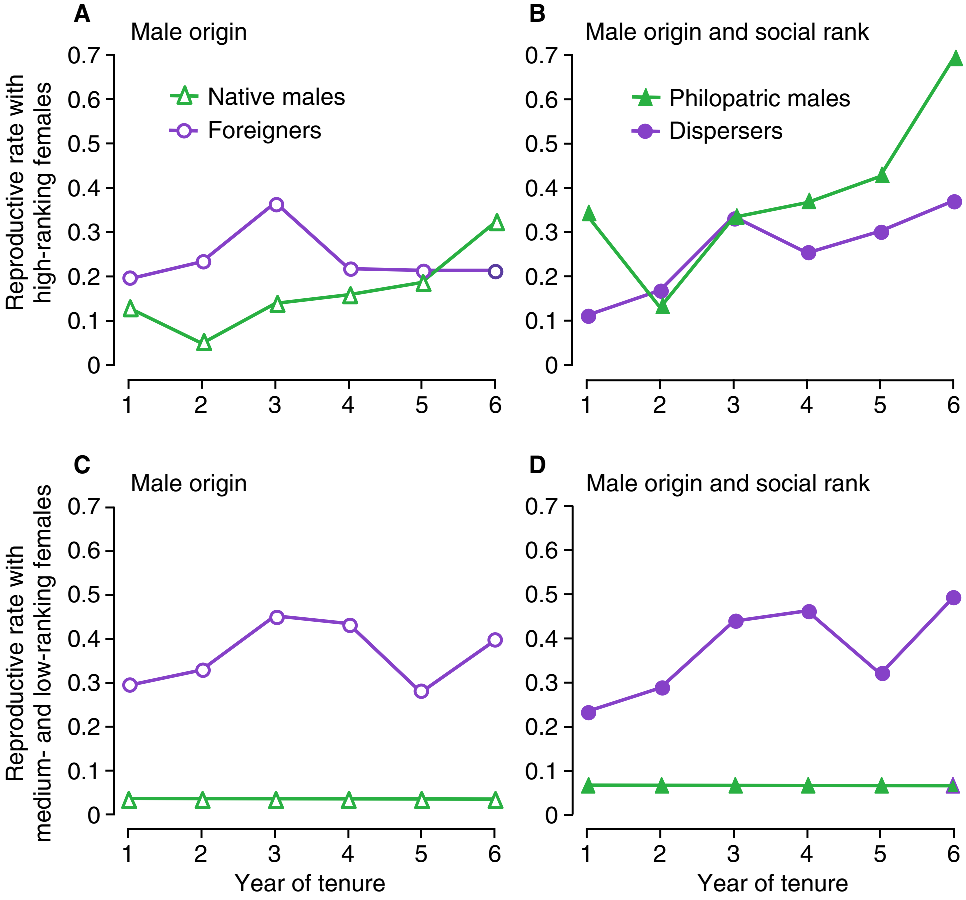

Fig. 3. Influence of male origin and combined effect of male origin and social rank on male reproductive rate. (**A** to **D**) Reproductive rate is the mean annual number of offspring sired with high-ranking females (A and B) and medium-ranking and low-ranking females (C and D). Male origin refers to whether males grew up in the chosen breeding clan (native males) or in another clan (foreigners). Data are back-transformed predictions derived from GLMMs that considered the covariates male origin, male social rank, maternal social rank, number of breeding partners at clan choice, year of tenure, and interaction between male origin and year of tenure. Values for the influence of male origin are adjusted means considering all other covariates at their population mean. Values for the combined influence of male origin and social rank are adjusted means computed as for male origin but considering the mean social rank of native males and foreigners, respectively, at each year of tenure. Because male origin and social rank represent the two main traits characterizing philopatric males and dispersers, their combined effect shows the difference in reproductive rate between philopatric males and dispersers.

partners that each clan contained on each date a male chose a clan (*25*). The distribution of clan qualities of natal clans differed from that of nonnatal clans ($\chi^2 = 152.4$, df = 7, $P < 0.001$), with natal clans being less likely to be of the highest quality and more likely to be of the lowest quality compared to nonnatal clans (Fig. 4A). As a result, philopatric males chose clans containing fewer likely female breeding partners (median = 5) compared to dispersers (median = 10; $U = 1621.5$, $P < 0.001$). To test whether philopatric males and dispersers applied different rules of clan choice, we compared the observed number of philopatric males that chose a clan of a given quality with the expected number under the assumptions that (i) philopatric males, similarly to dispersers, base their clan choice on the number of likely breeding partners and (ii) choosing the natal clan does not incur additional benefits or costs as compared to choosing a nonnatal clan. The expected number of (philopatric) males choosing their natal clan when it is of quality $\boldsymbol{i}$ is thereby equal to the product of the proportion of dispersers that chose a clan of quality $\boldsymbol{i}$ (Fig. 4C) and the proportion of clans of quality $\boldsymbol{i}$ that are natal clans (Fig. 4B, dark gray), multiplied by the total number of males observed to choose a clan ($n = 254$; Fig. 4D). The observed number of philopatric males ($n = 41$, 16.1% of all males that chose a clan) was twice as high as expected in all clan qualities ($n = 19.5$, 7.7%; Wilcoxon signed-rank test, $U = 36$, $P = 0.008$, $n = 8$ clan qualities; Fig. 4D). However, the relative distribution of philopatric males across the eight levels of clan quality did not differ from expectations ($\chi^2 = 0.81$, df = 7, $P = 0.99$; Fig. 4E).

Tenure in clan of first choice

Philopatric males were more likely to disperse from their first breeding clan (13 of 26 males with a monitoring period ≥6 years) than were dispersers (27 of 146; Fisher's exact test, odds ratio = 2.69, 95% CI = 1.12 to 6.26, $P = 0.021$), and they stayed for a shorter period in their clan of first choice (median from Kaplan-Meier survival analysis = 2.32 years, $n = 41$) than did dispersers (median = 6.03 years, $n = 213$; log-rank test, $\chi^2 = 12.4$, df = 1, $P < 0.001$; Fig. 5).

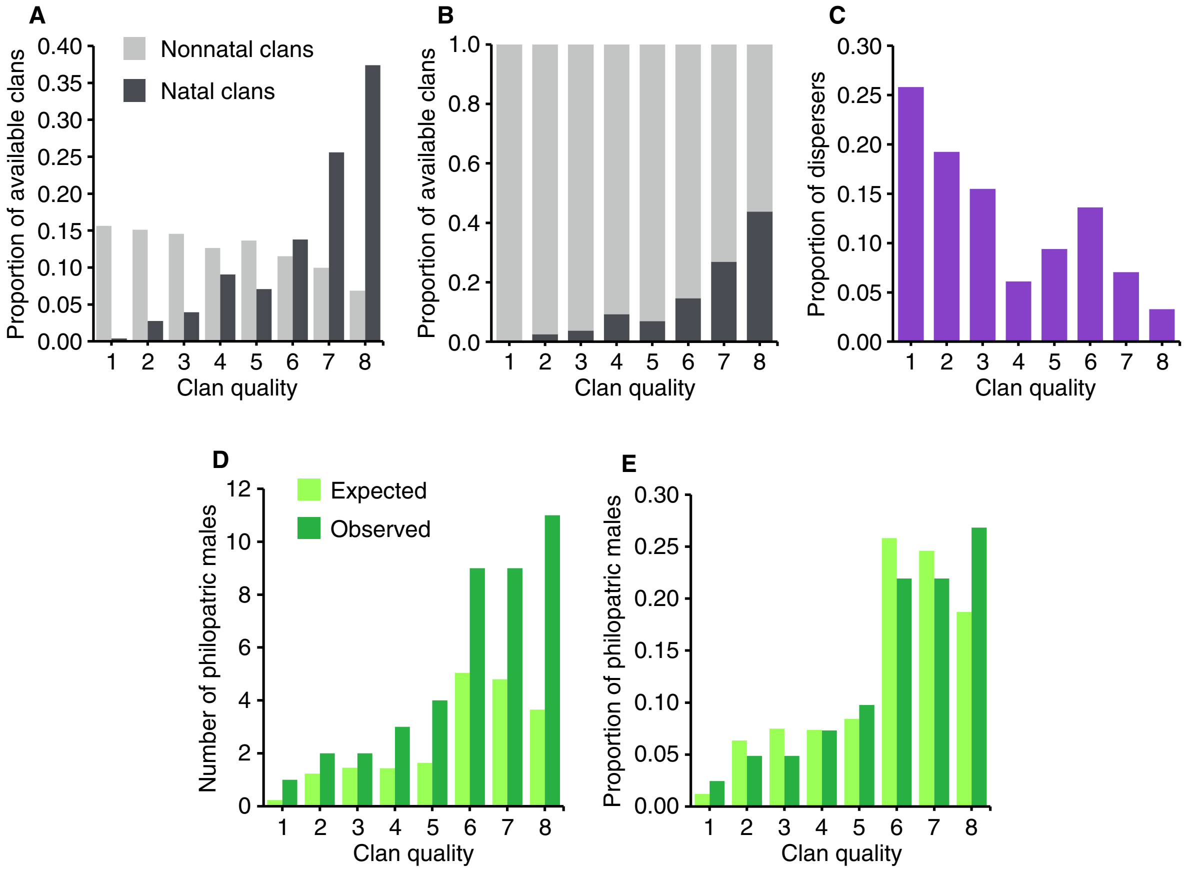

Fig. 4. Effect of clan quality on male clan choice and propensity of philopatry. (**A**) Distribution of natal and nonnatal clans across eight clan qualities as defined by the number of likely female breeding partners on the date a male chose a clan. Clan quality 1 corresponds to the clan with the highest number of likely breeding partners, and clan quality 8 corresponds to the clan with the lowest number of likely breeding partners. (**B**) Proportion of natal and nonnatal clans of each clan quality. (**C**) Distribution of clan choices by dispersers across the eight clan qualities. (**D**) Expected and observed number of philopatric males that chose each clan quality. (**E**) Expected and observed proportions of philopatric males across the eight clan qualities.

DISCUSSION

Our findings are inconsistent with the hypothesis that male philopatry is the result of high dispersal costs and a best-of-a-bad strategy pursued by phenotypically inferior males. Philopatric males chose clans containing fewer likely female breeding partners compared to dispersers, but they sired as many offspring as did dispersers and had survival after clan choice similar to dispersers. They also had mothers of similar social rank, suggesting that dispersal propensity was not influenced by the maternal environment during development and that philopatric males and dispersers were of similar phenotypic quality at clan choice. Our finding that philopatric males and dispersers chose a clan at a similar age further indicates that the process leading to philopatry or dispersal required a similar amount of time. The search and survival costs of prospection and settlement may indeed be low in our population of spotted hyenas and may not differ substantially between philopatric males and dispersers because both undertake short excursions to other clan territories before choosing a clan, dispersal distances are short, and recently settled dispersers frequently return to their natal clan territory and benefit from resuming their natal rank when feeding there (*25*, *29*).

Our results, instead, are consistent with the alternative hypothesis derived from habitat selection theory: The coexistence of philopatric males and dispersers results from all males applying similar rules of clan choice. Philopatric males distributed themselves similarly to dispersers when considering the quality of clans, indicating that they had similar preferences for clans of high quality, that is, clans with the largest number of potential breeding partners. Thus, philopatric males did not choose clans of lower average quality compared to dispersers because they were less choosy, but because natal clans were less likely to be of high quality than were nonnatal clans. The latter is a direct consequence of the rules female hyenas apply when choosing their mates (*25*); these rules imply that, in nonnatal clans, all females between 1 and 5 years of age on the date of male clan choice are likely to accept the male as a sire, whereas in natal clans, only females older than the male and younger than 5 years are likely breeding partners. Because males choose a clan at a mean age of 3.4 years, the number of likely breeding partners for a prospective philopatric male is reduced to females older than 3.4 years and younger than 5 years.

Although philopatric males chose clans containing fewer likely breeding partners, their fitness prospects were similar to those of

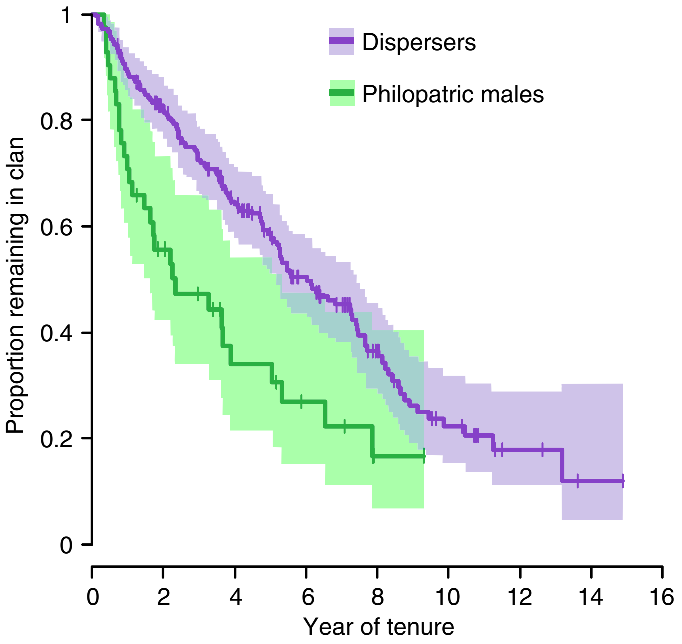

Fig. 5. Survivorship functions of the tenure of males in their first breeding clan. Curves correspond to the nonparametric Kaplan-Meier estimator of the proportion of philopatric males and dispersers with a total tenure longer than a given tenure. Data include complete tenures as exact values and tenures of males still alive at the end of the study as right-censored data (tick marks). Shaded areas represent 95% CIs.

dispersers. They sired fewer offspring than did dispersers with medium-ranking and low-ranking females, but because they occupied a high social rank throughout their tenure and social rank strongly increased reproductive success, they sired as many offspring as did dispersers. Moreover, as predicted, they started reproducing earlier than did dispersers and sired almost exclusively offspring with high-ranking females, a tactic that provides them with substantial short- and long-term fitness benefits because high-born offspring have a higher reproductive value than medium-born and low-born offspring; they survive better, benefit from "silver spoon" effects in terms of increased reproductive success, and contribute more to future generations than low-born offspring (*29*, *37*). In our study population, after only eight generations, a large majority of the living adult population (82% of 340 adults with known female ancestor) were descendants of high-ranking females present at the beginning of the study [for a similar result on female descendants in a single hyena clan in the Maasai Mara in Kenya, see Holekamp *et al.* (*38*)]. We predicted that such fitness benefits should increase the propensity of males choosing their natal clan as their breeding group. Consistent with this prediction, twice as many males chose philopatry as expected if philopatry did not yield additional benefits compared to dispersal. The shorter tenure of philopatric males additionally suggests that males regularly assess their fitness prospects and disperse as a secondary breeding-group choice when the fitness advantages associated with their high social rank decrease. These findings demonstrate that, in spotted hyenas, the coexistence of philopatric males and dispersers results from a single, continuous process of breeding-group selection driven by the distribution of group qualities across natal and nonnatal groups and the fitness benefits associated with the natal group. Our results are therefore consistent with a recent theoretical model that demonstrates that variation in dispersal distances among individuals of the same sex can emerge solely from the distribution and density of breeding partners when all individuals apply similar mate-based settlement rules (*19*).

Our results provide novel insights into the processes leading to philopatry and dispersal. By demonstrating that philopatric males can gain immediate, direct reproductive benefits, our study contrasts with most empirical studies on group-living mammals characterized by high dispersal biases. These studies reported that young philopatric males mainly gain indirect reproductive benefits through cooperative care of related offspring or delayed direct benefits after queuing for dominant breeder status (*39*–*42*). Most of these studies were conducted on male-dominated systems in which competition for females is high and access to high social rank and reproduction is correlated with male phenotypic traits such as age, body size, or fighting ability. We show here that in a promiscuous mating system, philopatry can provide similar fitness prospects to dispersal. Similar to systems where males and females are co-dominant (*43*), socially dominant hyena females can influence the competition among males and ensure, through social support (*31*), that their philopatric sons obtain a high social rank among sexually active males and its associated fitness benefits. These findings are consistent with the idea that fitness consequences of male philopatry depend on complex eco-evolutionary feedbacks and interactions between drivers of dispersal and male fitness, and the species' social and breeding system (*2*, *5*, *44*).

Our findings on male reproductive success differ from those of a previous study on spotted hyenas in the Maasai Mara in Kenya, which reported that philopatric males were less successful than dispersers (*32*). They further contradict the suggestions that philopatric male hyenas undergo physiological suppression (*45*, *46*) and are generally avoided by females as sires (*32*). These discrepancies may reflect real differences in hyena behavior and ecology between the two study populations, but they may also result from a difference in methodology. In contrast to our study, these previous studies used an age-based definition of philopatry, which resulted in the inclusion of males that were not yet sexually active and had not yet exercised breeding-clan choice (*32*, *34*). Such a definition will likely bias estimates of male investment in reproduction and reproductive success in favor of dispersers [as discussed in Bercovitch (*47*) and Höner *et al.* (*48*)]. Our results highlight the importance and benefit of treating not only the dispersal movement but also the dispersal destination and philopatry as a choice of a breeding group or habitat. This implies that dispersal and philopatry should be carefully defined (*9*, *49*), and the criteria applied should indicate reproductive investment (*47*, *48*). It also requires a consideration of the quality of potential breeding sites, including the natal site (*50*, *51*), and the identification of the ultimate and proximate factors driving male fitness. Only then will it be possible to assess whether differences in fitness and life history between philopatric individuals and dispersers result from differences in sexual maturity, their phenotype, the quality of the chosen breeding group, or the dispersal movement itself, and to understand the underlying processes leading to observed dispersal patterns (*48*, *50*, *52*–*55*).

Our study suggests that dispersal patterns within a sex may primarily depend on the likelihood that nonnatal groups provide higher fitness than the natal group, with the direction and magnitude of the bias depending on the nature of the drivers of individual fitness. In systems where the natal group systematically provides lower fitness than nonnatal groups, leaving the natal group may be of high adaptive value and males may even emigrate without prior prospection (*56*). This may drive the emergence of a dichotomous process of breeding-group selection in which individuals only choose between staying and emigrating, and of distinct philopatry and dispersal "syndromes" (*4*, *10*, *56*). By considering the quality of potential breeding groups, the choices of males, and the fitness outcome of their choices, we demonstrate that high dispersal biases can also emerge in systems in which natal groups can provide the highest fitness. We also demonstrate that philopatric males and dispersers may not necessarily represent two groups of individuals with distinct phenotypes and life histories. Our study illustrates the benefit of studying dispersal and philopatry within the broader framework of habitat selection theory to derive predictions on the causes and fitness consequences of breeding-group choices and dispersal patterns.

MATERIALS AND METHODS

Study design

All hyenas of the eight resident clans inhabiting the 250-km^2 floor of the Ngorongoro Crater (3°11′S, 35°34′E) in Tanzania were individually known and monitored between April 1996 and December 2014. Dispersal is strongly male-biased in this population, with approximately 85% of males leaving their natal clan and immigrating into another clan to breed, whereas females usually are philopatric (*25*, *57*). The population is genetically linked to other hyena populations (*58*), but most males born in one of the Crater clans choose to breed in a Crater clan. During the study period, 41 Crater-born males started their reproductive career in their natal clan, and 213 males dispersed to another Crater clan. An additional 54 Crater-born males that reached 2.7 years, the mean age at clan choice minus 1 SD (see Results), either died or emigrated out of the Crater, and 24 males immigrated into Crater clans from elsewhere. Clans contained a mean number of 53.5 ± 5.5 adult (≥24 months old) members at the end of the study period.

Clan choice and quality of clans

We defined breeding-clan selection as the behavioral processes that result in a biased investment (a choice) in social relationships and sexual behavior that may influence an individual's survival and reproduction [adapted from "habitat selection" (*10*)]. We defined philopatry and natal dispersal as the outcome of breeding-clan selection that led a male to choose and start his reproductive career in his natal clan or in another clan, respectively. We considered a male to have chosen a clan when he expressed sexual behavior toward females and invested in joining the social hierarchy of sexually active males in the natal clan ("philopatric" male) or another clan ("disperser") for at least 3 months (*29*). The date of clan choice was the date of first observation of such behavior for philopatric males and of first sighting in the new clan territory for dispersers, and defined the start of a male's tenure. We excluded males from the analyses that did not show any sign of sexual activity or had not met the 3-month criterion before their date of last sighting or the end of the study period.

We defined the quality of a breeding group as the social, demographic, and ecological characteristics of the group that influence the fitness of males, such as the number of unrelated breeding partners or competitors and food availability within the group territory [adapted from "habitat patch quality" (*59*)]. In spotted hyenas, male long-term fitness prospects and, thereby, clan quality are strongly influenced by the number of likely female breeding partners as defined by female mate-choice rules (*25*).

Social rank

Social ranks were assigned on the basis of the outcome of dyadic interactions using submissive responses. To compare social rank within and between clans when clan size differed, adult females and sexually active males of a clan were assigned a standardized rank by distributing ranks evenly between the highest (standardized rank +1) and lowest rank (standardized rank −1) in the hierarchy of adult females and sexually active males, respectively (*30*). Individuals with standardized ranks within the top, middle, and lower thirds of the total range were classified as high-ranking, medium-ranking, and low-ranking, respectively. Maternal social rank was calculated when sons were 2 years of age. Male social rank was calculated at the start of each year of tenure in the chosen clan.

Paternity assignment and reproductive success of males

Female spotted hyenas produce litters of one or two (very rarely three) cubs with no distinct breeding season (*33*). We collected tissue, hair, and fecal epithelium from 1246 hyenas, including 1101 offspring born during the study period. Samples were stored in ethanol or dimethyl sulfoxide salt solution until DNA extraction. Fluorescent primers were used to amplify nine polymorphic microsatellite loci (*60*). The mean number of alleles per locus was 11.9 (range, 7 to 16), the mean expected heterozygosity was 0.83, the total exclusionary power was 0.999, and the error rate was 0.44% and set at 1.0%.

Paternities were assigned using maximum likelihood methods as implemented in CERVUS 3.0 (*61*). Candidate fathers were determined on the basis of conception dates, which were calculated by subtracting a gestation period of 110 days from birth dates estimated from pelage characteristics, body size, locomotory abilities, behavioral development and position, and the shape and size of the ears of cubs (*62*–*64*). All philopatric males and dispersers that were clan members when a litter was conceived were considered to be candidate fathers. For 1048 offspring (95.2% offspring sampled), all candidate males were genetically typed; the mean proportion of typed candidate males was 0.99. Extra-clan paternity was very rare; 1064 offspring (96.6% sampled offspring) were sired by a philopatric or immigrant male of the clan. For the remaining 37 offspring, we performed a second analysis, which considered all adult males of the study population alive at conception as candidate fathers. For seven of these offspring (0.6% of all offspring), a male from another Crater clan was assigned paternity; for 30 offspring, no candidate male was assigned paternity at the 95% confidence level. To examine the influence of breeding-clan choice on male reproductive success, we restricted statistical analyses of reproductive success to offspring sired by philopatric males and dispersers with females from the chosen breeding clan. Male reproductive success was expressed as the annual number of offspring sired ("reproductive rate") during each of the first 6 years of tenure. A period of tenure of 6 years covers a substantial period of the reproductive career of males in a clan (see Results). Statistical analyses were restricted to paternities assigned at the 95% confidence level.

Ethical statement

Our study was approved by the scientific advisory board of the Tanzania Wildlife Research Institute, the Tanzania Commission for Science and Technology, the Ngorongoro Conservation Area Authority, and the

Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research in Berlin, the institute's equivalent of the Institutional Animal Care and Use Committee.

Statistical analyses

Statistical analyses were conducted using R software v.3.1.2 and associated packages (*65*). Data are means ± SD unless stated otherwise. The threshold for significance was set to 5%. We tested predictions assuming that philopatric males and dispersers represented two groups of individuals applying distinct strategies in two steps: (i) direct comparison of raw data using nonparametric Mann-Whitney *U* tests to characterize differences in key traits of hyena life history between philopatric males and dispersers and (ii) ordinary least-squares LMs and GLMMs to identify the variables influencing these traits.

Age at clan choice and tenure at first reproduction

We conducted LMs to examine the factors influencing the age at which male hyenas chose their first breeding clan and the tenure at which they sired their first offspring. We included the identity of the natal clan as a categorical covariate (eight levels) to control for clan specifics that may affect male condition or age at sexual maturity and thereby influence the timing of male clan choice, such as the number of clan members and prey abundance. We included the identity of the chosen clan as a categorical covariate (eight levels) to control for factors that may influence the date of first observation of social or sexual behaviors toward members of the clan and first sighting of a male in the chosen clan's territory, such as our monitoring effort and territory size. For the analysis of the age at which males chose their first breeding clan, we applied a natural logarithm transformation to the dependent variable "age" (in years).

Annual reproductive rate

We conducted three GLMMs to examine factors influencing male annual reproductive rate. We used the annual number of offspring sired by males as the dependent variable (i) with females of all social ranks, (ii) with high-ranking females, and (iii) with medium- and low-ranking females. We used the natural logarithm as the link function and the Poisson distribution as the probability distribution of the dependent variable [package *lme4* v.1.1.7 (*66*)]. Covariates included male origin [that is, whether the male had grown up in the chosen breeding clan (native) or in another clan (foreigner)], male social rank, maternal social rank, the number of likely female breeding partners at clan choice, the year of tenure as the categorical variable (six levels), and the interaction between male origin and year of tenure; male identity was included as a random factor and assumed to follow a Gaussian distribution. Because male tenure was categorized in 1-year periods, GLMMs were restricted to males with complete years of tenure; a male was considered to have completed a year of tenure when all offspring conceived in the male's chosen clan and sampled during that period were genetically typed. The model on male annual reproductive rate with medium- and low-ranking females failed to fit the interaction between male origin and year of tenure because of very low variation in the reproductive rate of native males over tenure. We therefore recoded, for native males only, the six levels associated with the factor "year of tenure" as a single level termed "native_year," thereby constraining the effect of tenure on the reproductive rate of native males to be constant over tenure (see Fig. 3, C and D). Because this factor then corresponded to the combined effect of male origin, year of tenure, and their interaction, we reran the GLMM discarding the covariate "male origin" to avoid redundant covariates. The recoding procedure allowed the model to compute regression coefficients separately for native males and foreigners; there is a single coefficient for native males and one coefficient for each of the six levels of years for foreigners (see table S5).

Regression coefficients for LMs and GLMMs were estimated by maximum likelihood using Laplace approximation (*66*). Significance of effects was assessed as the marginal contribution of each covariate to the full model by subtracting the likelihood of the reduced model without the specific covariate from the full model; *P* values were calculated using asymptotic LR tests [package *car* v.2.0.24 (*67*)] for LMs and parametric bootstrapped LR tests with 1000 simulations [package *pbkrtest* v.0.3.8 (*68*)] for GLMMs. Residuals of all LMs satisfied the assumptions of normal distribution [Lilliefors tests; package *nortest* v.1.0.2 (*69*)] and homogeneity of variances (residual plots). None of the GLMMs performed showed signs of overdispersion (Pearson residuals ratio), thereby conforming to the prerequisite for Poisson regressions or signs of multicollinearity between fixed effects (correlation matrices).

Male survival and tenure

Male survival and tenure were analyzed using the R package survival v. 2.37.7 (*70*). Differences in survival of philopatric males and dispersers after first clan choice were tested using Cox proportional hazards regressions with death or disappearance as the dependent variable. Individuals were considered to have died or disappeared when their dead body was found or when they were not sighted for at least 1 year. We assumed equal probability for both male categories to (re)disperse out of the Crater population. We used discrete time-event analysis on 1-year intervals to account for changes in male category (philopatric male versus disperser) over time and included males that were still alive at the end of the study (right-censored data). The assumption of proportional hazards over time was tested and validated using scaled Schoenfeld residuals. We compared male tenures in their first breeding clan using nonparametric Kaplan-Meier survivorship functions and the nonparametric log-rank test with (re)dispersal, death, or disappearance as the dependent variable, and tenures of males that were still alive and were still a member of their clan at the end of the study (right-censored data).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/3/e1501236/DC1

Table S1. The age at which male spotted hyenas chose their first breeding clan as a function of male origin (native or foreigner), maternal social rank, and identities of natal and chosen clans.

Table S2. The tenure at which male spotted hyenas sired their first offspring as a function of male origin (native or foreigner), maternal social rank, the number of likely breeding partner at clan choice, and the identity of the chosen clan.

Table S3. The total number of offspring sired each year by male spotted hyenas as a function of male origin (native or foreigner), male social rank, maternal rank, the number of likely breeding partners at clan choice, year of tenure, and the interaction between male origin and year of tenure.

Table S4. The number of offspring of high-ranking females sired each year by male spotted hyenas as a function of male origin (native or foreigner), male social rank, maternal social rank, the number of likely breeding partners, year of tenure, and the interaction between male origin and year of tenure.

Table S5. The number of offspring of medium- and low-ranking females sired each year by male spotted hyenas as a function of the combined effect of male origin and year of tenure, male social rank, maternal social rank, and the number of likely breeding partners.

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Supplementary Materials for

Why do some males choose to breed at home when most other males disperse?

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The PDF file includes:

Table S1. The age at which male spotted hyenas chose their first breeding clan as a function of male origin (native or foreigner), maternal social rank, and identities of natal and chosen clans.
Table S2. The tenure at which male spotted hyenas sired their first offspring as a function of male origin (native or foreigner), maternal social rank, the number of likely breeding partner at clan choice, and the identity of the chosen clan.
Table S3. The total number of offspring sired each year by male spotted hyenas as a function of male origin (native or foreigner), male social rank, maternal rank, the number of likely breeding partners at clan choice, year of tenure, and the interaction between male origin and year of tenure.
Table S4. The number of offspring of high-ranking females sired each year by male spotted hyenas as a function of male origin (native or foreigner), male social rank, maternal social rank, the number of likely breeding partners, year of tenure, and the interaction between male origin and year of tenure.
Table S5. The number of offspring of medium- and low-ranking females sired each year by male spotted hyenas as a function of the combined effect of male origin and year of tenure, male social rank, maternal social rank, and the number of likely breeding partners.

Table S1. The age at which male spotted hyenas chose their first breeding clan as a function of male origin (native or foreigner), maternal social rank, and identities of natal and chosen clans. Shown are the regression coefficients and standard errors (SE) in natural log units, t ratios and their corresponding *P*-values for each predictor variable. Regression coefficients were estimated by ordinary least squares linear regression (whole model: Likelihood Ratio = 1.43, d.f. = 16, *P* = 0.009, *n* = 214 males) and indicate the change in age at clan choice relative to the intercept and the reference level for the categorical variables (male origin: foreigner, natal clan: clan A, chosen clan: clan A).

Variable	Coefficient	SE	t ratio	*P*
Intercept	1.20	0.06	21.36	< 0.001
male origin native	-0.01	0.04	-0.26	0.79
maternal social rank	0.04	0.03	1.57	0.12
natal clan E	0.09	0.07	1.25	0.21
natal clan F	-0.12	0.07	-1.76	0.08
natal clan L	0.05	0.06	0.95	0.34
natal clan M	-0.01	0.06	-0.13	0.89
natal clan N	0.14	0.07	1.96	0.05
natal clan S	0.11	0.07	1.57	0.12
natal clan T	0.08	0.11	0.75	0.46
chosen clan E	-0.09	0.06	-1.46	0.15
chosen clan F	0.09	0.06	1.41	0.16
chosen clan L	-0.06	0.05	-1.10	0.27
chosen clan M	-0.11	0.06	-1.94	0.05
chosen clan N	-0.01	0.06	-0.13	0.90
chosen clan S	-0.01	0.05	-0.12	0.91
chosen clan T	-0.01	0.08	-0.16	0.87

Table S2. The tenure at which male spotted hyenas sired their first offspring as a function of male origin (native or foreigner), maternal social rank, the number of likely breeding partner at clan choice, and the identity of the chosen clan. Shown are the regression coefficients and standard errors (SE), t ratios and their corresponding *P*-values for each predictor variable. Regression coefficients were estimated by ordinary least-squares linear regression (whole model: Likelihood Ratio = 73.33, d.f. = 10, $P < 0.001$, $n = 136$ males) and indicate the change in tenure at which males sired their first offspring relative to the intercept and the reference level for the categorical variables (male origin: foreigner, chosen clan: clan A).

Variable	Coefficient	SE	t ratio	*P*
Intercept	2.68	0.50	5.33	< 0.001
male origin native	-1.21	0.38	-3.23	0.002
maternal social rank	-0.84	0.19	-4.41	< 0.001
number of likely breeding partners	-0.02	0.03	-0.77	0.44
chosen clan E	-0.35	0.45	-0.79	0.43
chosen clan F	-0.58	0.48	-1.21	0.23
chosen clan L	-0.25	0.41	-0.61	0.54
chosen clan M	-0.30	0.42	-0.72	0.47
chosen clan N	0.38	0.50	0.75	0.45
chosen clan S	-1.44	0.49	-2.93	0.004
chosen clan T	-0.72	0.72	-0.99	0.32

Table S3. The total number of offspring sired each year by male spotted hyenas as a function of male origin (native or foreigner), male social rank, maternal rank, the number of likely breeding partners at clan choice, year of tenure, and the interaction between male origin and year of tenure. Shown are the regression coefficients and standard errors (SE) in natural log units, z ratios and their corresponding *P*-values for each predictor variable. Regression coefficients were estimated by generalized linear mixed model with the natural logarithm as link function and Poisson and Gaussian distributions as the assumed probability distributions for the dependent variable and the random effect (male identity, variance = 0.33), respectively (whole model: Likelihood Ratio = 71.52, *P* = 0.001, *n* = 181 males). Coefficients indicate the change in the number of offspring sired each year by males relative to the intercept and the reference level for the categorical variables (male origin: foreigner, year of tenure: year 1).

Variable	Coefficient	SE	z ratio	*P*
Intercept	-0.59	0.20	-2.93	0.003
male origin native	-0.65	0.39	-1.69	0.094
male social rank	0.62	0.16	3.84	< 0.001
maternal social rank	0.22	0.11	1.90	0.058
number of likely breeding partners	-0.002	0.01	-0.18	0.86
year of tenure 2	0.14	0.17	0.86	0.39
year of tenure 3	0.53	0.17	3.12	0.002
year of tenure 4	0.27	0.20	1.36	0.18
year of tenure 5	0.03	0.23	0.11	0.91
year of tenure 6	0.20	0.25	0.80	0.42
native * year of tenure 2	-1.38	0.66	-2.09	0.036
native * year of tenure 3	-0.58	0.50	-1.16	0.25
native * year of tenure 4	-0.25	0.57	-0.44	0.66
native * year of tenure 5	0.14	0.59	0.24	0.81
native * year of tenure 6	0.44	0.61	0.73	0.47

Table S4. The number of offspring of high-ranking females sired each year by male spotted hyenas as a function of male origin (native or foreigner), male social rank, maternal social rank, the number of likely breeding partners, year of tenure, and the interaction between male origin and year of tenure. Shown are the regression coefficients and standard errors (SE) in natural log units, z ratios and their corresponding *P*-values for each predictor variable. Regression coefficients were estimated by generalized linear mixed model with the natural logarithm as link function and the Poisson and Gaussian distributions as the assumed probability distributions for the dependent variable and the random effect (male identity, variance = 0.73), respectively (whole model: Likelihood Ratio = 56.97, $P <$ 0.001, n = 181 males). Coefficients indicate the change in the number of high-ranking offspring sired each year by males relative to the intercept and the reference level for the categorical variables (male origin: foreigner, year of tenure: year 1).

Variable	Coefficient	SE	z ratio	*P*
Intercept	-1.60	0.32	-5.03	< 0.001
male origin native	-0.40	0.53	-0.77	0.45
male social rank	0.95	0.24	3.99	< 0.001
maternal social rank	0.21	0.17	1.20	0.23
number of likely breeding partners	-0.00007	0.02	-0.003	0.99
year of tenure 2	0.19	0.26	0.71	0.47
year of tenure 3	0.64	0.26	2.43	0.015
year of tenure 4	0.12	0.31	0.38	0.70
year of tenure 5	0.10	0.33	0.30	0.76
year of tenure 6	0.11	0.37	0.26	0.78
native * year of tenure 2	-1.14	0.71	-1.61	0.11
native * year of tenure 3	-0.57	0.59	-0.97	0.33
native * year of tenure 4	0.08	0.65	0.13	0.90
native * year of tenure 5	0.26	0.67	0.40	0.70
native * year of tenure 6	0.81	0.71	1.15	0.25

Table S5. The number of offspring of medium- and low-ranking females sired each year by male spotted hyenas as a function of the combined effect of male origin and year of tenure, male social rank, maternal social rank, and the number of likely breeding partners. Shown are the regression coefficients and standard errors (SE) in natural log units, z ratios and their corresponding P-values for each predictor variable. Regression coefficients were estimated by generalized linear mixed model with the natural logarithm as link function and the Poisson and Gaussian distributions as the assumed probability distributions for the dependent variable and the random effect (male identity, variance = 0.33), respectively (whole model: Likelihood Ratio = 45.89, $P < 0.001$, $n = 181$ males). Coefficients indicate the change in the number of medium- and low-ranking offspring sired each year by males relative to the intercept and the reference level for the categorical variable (foreigner in year of tenure 1).

Variable	Coefficient	SE	z ratio	*P*
Intercept	-1.19	0.25	-4.69	< 0.001
native_year	-2.00	0.58	-3.45	< 0.001
foreigner_year of tenure 2	0.12	0.22	0.53	0.60
foreigner_year of tenure 3	0.43	0.23	1.93	0.054
foreigner_year of tenure 4	0.39	0.26	1.53	0.13
foreigner_year of tenure 5	-0.05	0.31	-0.16	0.87
foreigner_year of tenure 6	0.30	0.33	0.91	0.36
male social rank	0.38	0.21	1.82	0.069
maternal social rank	0.24	0.14	1.72	0.086
number of likely breeding partners	-0.005	0.02	-0.29	0.77

CHAPTER 4

Determining hormone metabolite concentrations when enzyme immunoassay accuracy varies over time

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Detailed contributions:

The concept of the study was developed by Eve Davidian (ED), Sarah Benhaiem (SB), Oliver Höner (OH), Heribert Hofer (HH), and Martin Dehnhard (MD). The cortisol-3-CMO enzyme immunoassay was initially developed by MD, and was physiologically and biologically validated by MD, SB, HH, and Marion East. Faecal samples were collected by OH, ED, and Bettina Wachter. Faecal sample freeze-drying, metabolite extraction, and measurements of concentrations in cortisol metabolites were conducted by technical assistants (Heidrun Barleben, Sylvia Schultz van Endert and Katrin Paschmionka), under the supervision of ED and MD. ED and OH implemented the method to identify groups of measurement of different accuracy (cluster analysis). ED conceptualized the method and criteria to assess the predictive performance of the model and to estimate the minimum number of samples required to apply the standardization method. Alexandre Courtiol (AC) implemented these criteria into a program in R software to quantify and plot the predictive performance of the model with varying sample size. Statistical analyses were conducted by ED and SB under the guidance of AC. The manuscript was primarily written by ED and SB, was extensively edited by OH and AC, and further commented on by HH and MD.

ABSTRACT

1. Enzyme immunoassays (EIAs) are widely used to quantify concentrations of hormone metabolites. Modifications in laboratory conditions may affect the accuracy of metabolite concentration measurements and lead to misinterpretations when results of different accuracy are combined for a statistical analysis. This issue is of great relevance to studies in behavioural and evolutionary ecology because these usually aim at understanding how hormone concentrations vary between individuals, environments or experimental conditions.

2. We present a method based on re-assaying a subset of samples to standardize hormone metabolite concentrations when changes in EIA accuracy occur. We used glucocorticoid metabolite concentrations (fGMCs) measured in faeces of spotted hyaenas (*Crocuta crocuta*) between 2011 and 2013 with a previously validated EIA. Changes in accuracy were assessed by monitoring the metabolite concentration of faecal control 'pools' that were systematically assayed with faecal samples. A cluster analysis on these pools identified two distinct sample sets with different EIA accuracy; 'Cluster 1' and 'Cluster 2'. We then re-assayed all samples of Cluster 1 (n = 138) with an EIA accuracy similar to that of Cluster 2 and fitted a linear regression to the remeasured fGMCs against the initial fGMCs to predict fGMCs in Cluster 2. To determine the minimum number of samples to re-assay that allows reliable predictions, we assessed the variation in the quality of model predictions by fitting linear regressions on decreasing numbers of re-assayed samples. This revealed that re-assaying 27 samples would be sufficient to generate reliable predictions considering our data set.

3. To test the robustness of our method, we fitted a new linear regression to 27 randomly chosen samples and used its equation to standardize all fGMCs of Cluster 1. The standardized fGMCs were similar to the remeasured fGMCs, and the regression on 27 samples was as effective at standardizing fGMCs as the regression fitted on the complete data set.

4. Our standardization method permits the combination of results of different accuracy. It is a simple and reliable alternative to the costly, time-consuming and often impractical re-assaying of complete sample sets that can be applied to a wide variety of species and sample types.

INTRODUCTION

Methods to measure concentrations of steroid hormone metabolites in urine and faeces have become an essential part of studies in evolutionary ecology and conservation. They have been applied to many taxa to investigate key topics such as the interplay between steroid hormones and social or sexual behaviour (Rasmussen et al. 2008; Benhaiem et al. 2013) and the physiological response of endangered species to disturbance (Rolland et al. 2012). Because the collection of urine and faeces does not involve potentially stressful procedures such as the manipulation or immobilisation of study animals, these methods are particularly useful to monitor adrenocortical activity over time and for studies on free-ranging animals (Hofer & East 1998; Touma & Palme 2005; Landys, Goymann & Slagsvold 2011; Rolland et al. 2012; Benhaiem et al. 2013).

Hormone metabolite concentrations are most commonly quantified using enzyme immunoassays (EIAs) (Touma & Palme 2005). In indirect, competitive EIAs, the metabolites in faecal or urine samples compete with a known amount of tracer (e.g. a steroid hormone conjugated with a peroxidase enzyme) for the binding sites of a hormone-specific antibody. The proportion of bound tracer generates a ‘response’, which is read photometrically and expressed as optical density. The metabolite concentration in a sample is then quantified by relating the optical density to a calibrated dose–response curve generated by standards of known hormone concentration (Wild 2013). Because the chemical structure of metabolites and their binding affinity towards the antibody usually differs from that of their native hormone contained in standards and tracer, there often is a bias between measured and ‘true’ metabolite concentration; this bias defines the ‘accuracy’ of an EIA (Wild 2013). Such a bias is negligible if it remains constant for all assayed samples and if, as in most studies in behavioural and evolutionary ecology, the main interest lies in relative differences in concentrations between individuals, environments or experimental conditions (Lynch et al. 2003; Brown, Walker & Steinman 2004). If, however, EIA accuracy substantially changes during the course of the study, measured metabolite concentrations in samples are not directly comparable and combining them in statistical models would lead to misinterpretations and erroneous conclusions.

Changes in EIA accuracy may occur for various reasons. EIAs involve binding reactions that are sensitive to laboratory conditions such as room temperature and exposure to light during incubation (responsible for ‘edge’ or ‘well-to-well’ effects; Watson et al. 2013). Other potential sources of variation in binding reactions are modifications in protocols such as changes in the concentration of antibody and tracer, replacement of the antibody, standards or other reagents when they are used up, expire or when commercial kits are discontinued, changes of equipment, and switches in laboratory personnel (Shekarchi et al. 1984; Jones et al. 1995; Noble et al. 2008; Wasser et al. 2010; Watson et al. 2013). Because the native hormone in standards and tracer and metabolites in samples differ in their chemical properties, their binding reactions may be affected differently by variation in laboratory protocol and conditions, thereby inducing changes in the accuracy of metabolite concentrations (Watson et al. 2013).

To track changes in EIA accuracy and other characteristics of EIA performance, control parameters such as the responses in blank wells, the standard concentrations associated with relative binding sensitivities (i.e. concentrations at 10, 20, 50, 80 and 90% of binding) and metabolite concentrations in urine or faecal control solutions or ‘pools’ are routinely monitored (Brown, Walker & Steinman 2004; Wild 2013). Pools are commonly used to assess the intra-assay and interassay repeatability or ‘precision’ of measurements of metabolite concentrations. Substantial changes in the concentration of pools additionally indicate changes in the relationship between measurements of metabolites and standards, that is, changes in EIA accuracy (Gill, Hayes & Sluss 2003). One possibility to avoid non-comparable results due to changes in accuracy is to (re-)assay all samples together and within a short period of time. Re-assaying large data sets for each new research question, however, is costly in time, manpower, sample material and money and may not always be feasible, for example when samples are depleted.

Here, we present a method to standardize results when changes in EIA accuracy occur, based on the re-assaying of a subset of samples. We establish this method using glucocorticoid metabolite concentrations measured in faeces (fGMCs) of spotted hyaenas (*Crocuta crocuta*) collected in the Ngorongoro Crater, Tanzania, as part of a long-term research project (Höner et al. 2007, 2010). We demonstrate that our method effectively standardizes metabolite concentrations and allows comparison of measurements obtained when EIA accuracy varies.

METHODS AND RESULTS

Collection and treatment of faecal samples

We collected 483 faecal samples from 272 free-ranging spotted hyaenas between 2002 and 2013. Faeces were collected immediately after defaecation, mixed, subsampled and stored in liquid nitrogen until transported to Germany on dry ice where they were stored at -80°C until further processing. Faecal subsamples were freeze-dried (for 49–70 h) with a Lyovac-GT2 lyophilisator (Hürth, Germany). Aliquots of 0.1 g were extracted with 0.9 mL of 90% methanol for 30 min, centrifuged, and the supernatant (typically 0.7 mL) diluted 1:1 with distilled water. Faecal samples and extracts were stored at -80°C between treatments.

Assay of faecal samples

Faecal extracts were assayed in three batches by two technicians; one technician assayed extracts in July 2011 (n = 71 extracts, 5 plates) and September 2011 (n = 67 extracts, 5 plates) and the other in July 2013 (n = 345 extracts, 13 plates). We quantified fGMCs using an 'in-house' cortisol-3-CMO competitive EIA that was validated for spotted hyaenas and demonstrated a high affinity of the antibody with cortisol metabolites, the ability of the antibody to measure natural fluctuation in metabolite concentrations and a high precision of measurement (Benhaiem et al. 2012). We used microtitre plates coated with a polyclonal antibody raised in rabbits against cortisol-3-CMO-BSAand cortisol- 3-CMO-peroxidase as tracer (for more details on EIA protocol, see Benhaiem et al. 2012). Calibrated standard curves were prepared by serial 1:2 dilutions of a cortisol stock solution and ranged from 0.2 to 100 pg 20 μL^{-1}. Calibration curves were fitted using Akima's spline interpolation (Akima 1970). The approximately linear range of the calibration curve (i.e. the section between 20 and 80%of binding of the tracer or 'binding sensitivity') was used to estimate fGMCs in samples using Magellan software (version 2.6; Tecan Group Ltd., Männedorf, Switzerland). Faecal samples with concentrations exceeding this range (typically > 25 pg 20 μL^{-1}) were diluted to provide precise quantification of metabolite concentrations. Final fGMCs were obtained by multiplying the measured raw fGMCs by their corresponding factor of dilution and expressed as ng g^{-1} of dry faecal matter.

We used two faecal control pools with relatively high and low metabolite concentrations (hereafter: 'high pool' and 'low pool') to monitor intra-assay and interassay precision and potential changes in EIA accuracy. Stock solutions of pools and standards were renewed several times (but never simultaneously) during the course of the study. Coefficients of variation (CV) between old and new stocks never exceeded 10%, thus complying with the commonly accepted interassay coefficient of variation ($CV_{interassay}$) of 20% (for details on this criterion, see the section on EIA performance and for the CV formula, see Appendix S1 in Supporting Information), and confirming that renewals of stocks were not associated with changes in pool concentrations nor with shifts in EIA binding sensitivity. Assay plates were subdivided following a design that was constant throughout the study, with specific wells assigned to standard solutions, pools, blank controls, and faecal extracts, respectively. All extracts and controls were assayed in duplicate and, as typically recommended (Wild 2013), measurements were only accepted when duplicated values did not differ by more than 5% from their mean (i.e.$CV_{intra\text{-}assay} \leq 5\%$). The concentrations of antibody and tracer were changed during the course of the study, but all other parameters of the experimental procedure and equipment were maintained constant.

Data analysis

Statistical procedures were performed using R software version 3.1.0 (R Development Core Team 2013). Results are quoted as mean ± standard deviation (SD), probabilities are for two-tailed tests, the threshold for significance was set at 5%, and 95% percentile confidence intervals (CI95%) were calculated using a bootstrap method with 100 000 iterations (R package ‘boot’; Canty & Ripley 2014). For ordinary least squares (OLS) linear regressions, including analyses of covariance (ANCOVA), the distribution of residuals did not significantly deviate from normality (Shapiro–Wilk tests) and the variances were homoscedastic (Breusch-Pagan tests and residuals plots; R package ‘car’; Fox & Weisberg 2011).

EIA performance during the study period

To assess whether the samples assayed during the entire study had comparable fGMCs, we calculated the $CV_{interassay}$ of the fGMCs of the pools across all 23 plates. The $CV_{interassay}$ of pools (mean = 58.4 ± 20.2%) exceeded the commonly applied criterion of 20% (e.g. Goymann et al. 1999; Bales et al. 2005; Ganswindt et al. 2005; Behie, Pavelka & Chapman 2010), indicating substantial variation in the accuracy ofmetabolitemeasurements during the study.

We then assessed separately for each of the three batches EIA precision, stability of the accuracy of measurements, analytical sensitivity, quantitative resolution and binding sensitivity. $CV_{intra-assay}$ and $CV_{interassay}$ of pools did not exceed 5%and 20%, respectively, indicating that the precision of the EIA was high and the accuracy remained stable within each batch of measurements (see Appendix S1 in Supporting Information). The results also indicated that the EIA maintained a high analytical sensitivity and quantitative resolution throughout the study. The binding sensitivity of the EIA was similar in July 2011 (range of standard concentrations at 10 – 90% of binding: 0.5 – 28.9 pg 20 µL^{-1}) and September 2011 (range: 0.6 – 30.5 pg 20 µL^{-1}) but lower in July 2013 (range: 0.9 – 40.5 pg 20 µL^{-1}; Fig. 1).

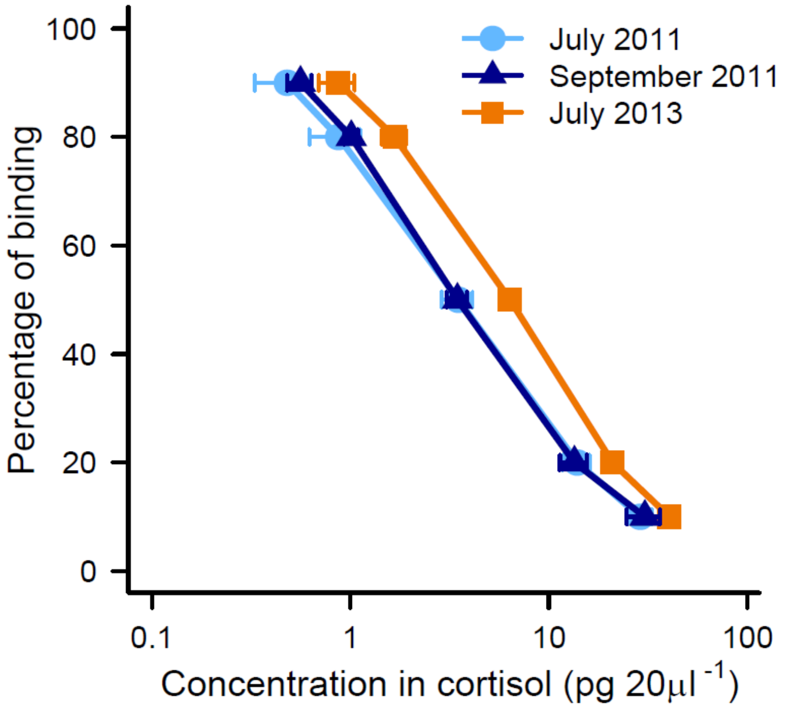

Fig. 1. Relationship between the percentage of binding of the tracer and measured cortisol concentration in standards. Symbols correspond to the mean SD concentration in cortisol at 10, 20, 50, 80 and 90% of binding of the tracer, for July 2011 (n = 5 standard curves), September 2011 (n = 5 standard curves) and July 2013 (n = 13 standard curves).

We also tested for interference with non-antigenic material in samples because such 'matrix effects' can disrupt the relationship between measurements of metabolites and hormone standards when faecal extracts are diluted. We applied two tests of parallelism which compared the slope of the calibration curve with that of the displacement curve obtained from serial dilutions of faecal extracts (Kemeny & Challacombe 1988). One test was performed in July 2011 using two faecal samples (‘A’ and ‘B’) that were extracted in 2011 and a second test was performed in July 2013 using two faecal samples (‘C’ and ‘D’) that were extracted in 2011 and 2012. Parallelism was validated for all four faecal extracts (ANCOVA, P-value for comparison of the slopes: P = 0.08 for extract A; P = 0.86 for B; P = 0.15 for C and P = 0.51 for D), demonstrating that there were no matrix effects on our measurements and that the bias between measurements of metabolites and standards was constant throughout the range of dilution of faecal extracts.

Establishment of the standardization procedure

The standardization procedure consists of (i) identifying ‘clusters’ of samples assayed with similar EIA accuracy and assigning the reference cluster, (ii) choosing and re-assaying a subset of samples with the EIA accuracy of the reference cluster, (iii) modelling the relationship between initial and remeasured metabolite concentrations, (iv) testing the predictive performance of the model and (v) standardizing the metabolite concentrations of all samples from the cluster. The following sections detail how we established the method using our data set on fGMCs in spotted hyenas.

Identifying clusters of samples assayed with similar EIA accuracy and assigning the reference cluster

To identify plates that contain pools of similar concentrations (i.e. similar accuracy) and determine which and how many faecal samples may need to be re-assayed and standardized, we conducted a cluster analysis on all measurements of fGMCs of the high and low pools simultaneously. We performed a hierarchical clustering using Ward’s agglomeration method on the dissimilarity matrix of Euclidean distances between the fGMCs of pools from the 23 plates (R core package ‘stats’;Ward 1963; Murtagh & Legendre 2014). This analysis identified two distinct clusters, referred to as Cluster 1 and Cluster 2 (Fig. 2). Cluster 1 comprised the 10 plates (n = 138 samples) assayed in July 2011 and September 2011 and Cluster 2 the 13 plates (n = 345 samples) assayed in July 2013. An alternative analysis conducted on the fGMCs of each pool separately revealed similar results. To verify that each cluster conformed to the generally accepted interassay variation in precision and accuracy of CV*interassay* ≤ 20%, we calculated the CV*interassay* of the pools in Cluster 1 and Cluster 2. The CV*interassay* of pools in the two clusters each conformed to the level of acceptance, indicating a stable EIA accuracy within each cluster (Fig. 2). We assigned Cluster

2 as the reference cluster for the re-assaying of samples and standardization of fGMCs because at the time of reassaying EIA accuracy corresponded to that of Cluster 2 (see following section). The fGMCs of low and high pools increased by a factor of 7.5 and 2.7, respectively, between Cluster 1 and Cluster 2.

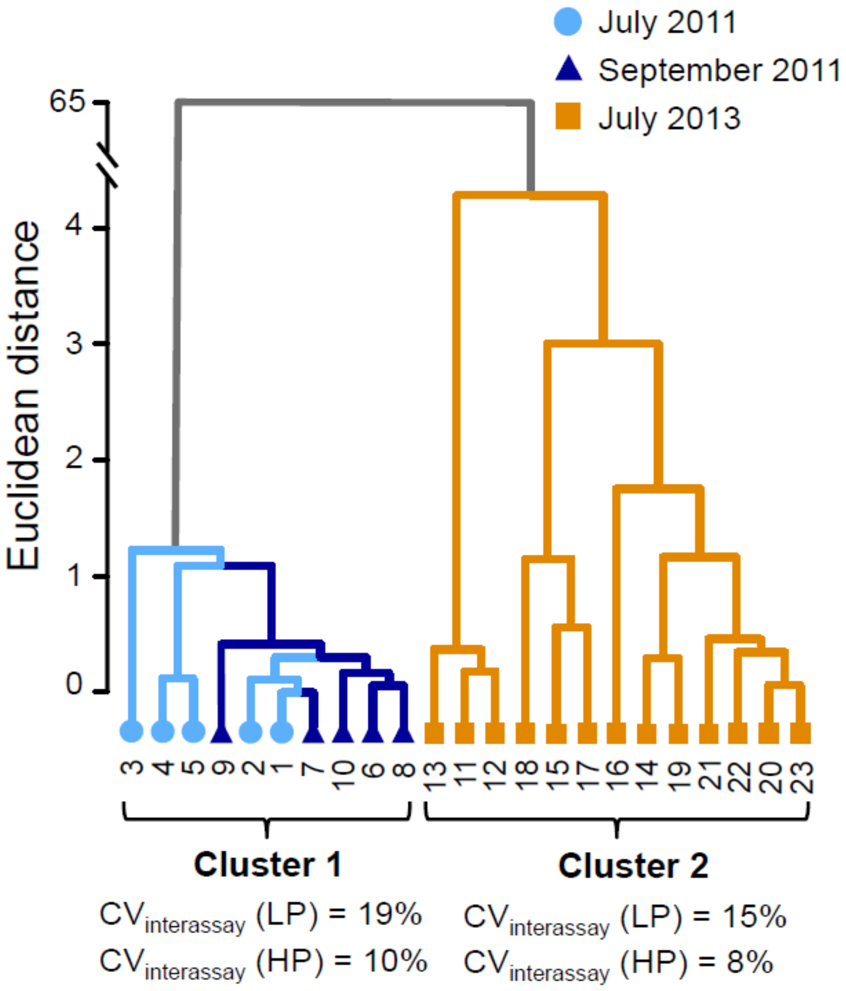

Fig. 2. Dendrogram showing the hierarchical clustering of the concentration of faecal pools assayed on 23 plates. The two clusters of faecal pools identified by the analysis are referred to as Cluster 1 and Cluster 2. $CV_{interassay}$ (LP) and $CV_{interassay}$ (HP) correspond to the interassay coefficient of variation for the low and high pool, respectively. Numbers (from1 to 23) below the dendrogram refer to the code of the plate.

Re-assaying samples with the EIA accuracy of the reference cluster

To establish our method, we re-assayed all 138 faecal samples of Cluster 1 within a few days after assaying the 345 samples of Cluster 2, using the same solutions of standards and pools, and applying the same EIA protocol as for Cluster 2. Optical densities of standards and concentrations of pools run with the re-assayed samples were similar to those of standards and pools run in Cluster 2 ($CV_{interassay} < 20\%$), confirming a stable EIA accuracy and binding sensitivity between Cluster 2 and reassaying. To avoid errors associated with different extraction procedures and dilutions of sample extracts, all re-assays were performed using the same sample extracts and dilutions as in Cluster 1.

Modelling the relationship between initial and remeasured concentrations using the complete data set

We modelled the relationship between the fGMCs initially measured in Cluster 1 ($fGMC_{initial}$, as x) and the fGMCs remeasured with the accuracy of Cluster 2 ($fGMC_{remeasured}$, as y) using an OLS linear regression on raw measurements, that is, before multiplying fGMCs by their corresponding dilution factor (for a comparison with an alternative linear model, see Appendix S1 in Supporting Information). The resulting equation was as follows:

$$fGMC_{remeasured} = 4.22 + 1.33 \times fGMCinitial \qquad \text{eqn 1}$$

This model accounted well for the variation in remeasured fGMCs (adjusted $r^2 = 0.72$; n = 138).

Cross-validating the model using the complete data set

We assessed the predictive performance of themodel using a cross-validation procedure (R package ‘DAAG’; Maindonald & Braun 2014) that divided the data set into three subsets of equal size. Alternately, two subsets were grouped and used as ‘training’ sets to fit an OLS linear regression while the remaining subset was used as a ‘test’ set to assess the reliability of predictions on an independent subset of samples.

Following our tolerated variation in precision and accuracy of repeated measurements of $CV_{interassay} \leq 20\%$, we considered model predictions to be reliable if the difference between predicted fGMCs and their matched remeasured fGMCs did not exceed 20% (i.e. $CV_{fit} \leq 20\%$).We further considered amodel to have a satisfactory predictive performance when at least 70% of samples had a CV_{fit} ≤ 20%. The cross-validation showed that 86.2% of samples (120 out of 138 samples) conformed to our criterion of CV_{fit}.

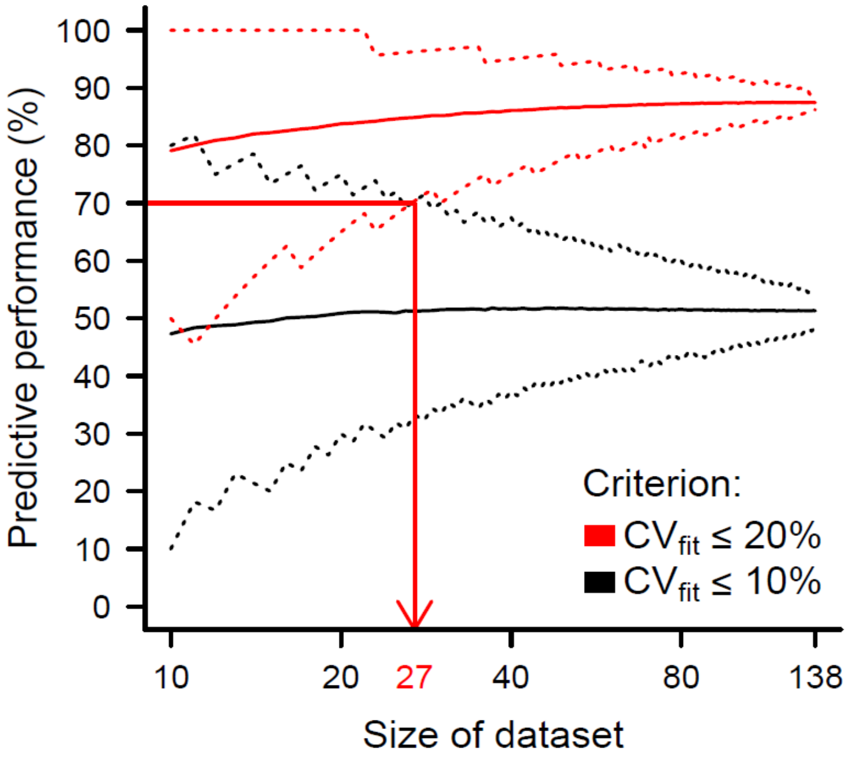

Fig. 3. Variation in the predictive performance of OLS linear regressions with decreasing size of the data set. Results indicate the mean (solid lines) and 95% confidence interval (dotted lines) percentage of faecal samples with a coefficient of variation (CV_{fit}) within the boundaries of CV_{fit} ≤20% and CV_{fit} ≤10%, calculated with 10 000 simulations. The red line with arrow indicates the smallest number of samples to re-assay (here, n = 27) to obtain at least 70% of samples with a CV_{fit} ≤20%. The x-axis is displayed on a logarithmic scale.

Estimating the minimum number of samples to re-assay

To estimate the minimum number of samples required to obtain reliable predictions, we generated data sets of decreasing size (i.e. from 138 to 10 samples) by choosing randomly, without replacement, a given number of faecal samples among the complete data set of 138 samples. We fitted and cross-validated OLS linear regressions on these data sets. The random sampling, model fitting and cross-validation procedures were reiterated 10 000 times for each data set. Results of the cross-validation of these models are illustrated in Fig. 3 for two different criteria of prediction reliability (i.e. CV_{fit} ≤ 20% and CV_{fit} ≤ 10%; see also Table S2 in Appendix S1 in Supporting Information). The smallest data set to reach our threshold formodel acceptance (i.e. 70% of CVfit ≤.20%) was 27 samples (Fig. 3).

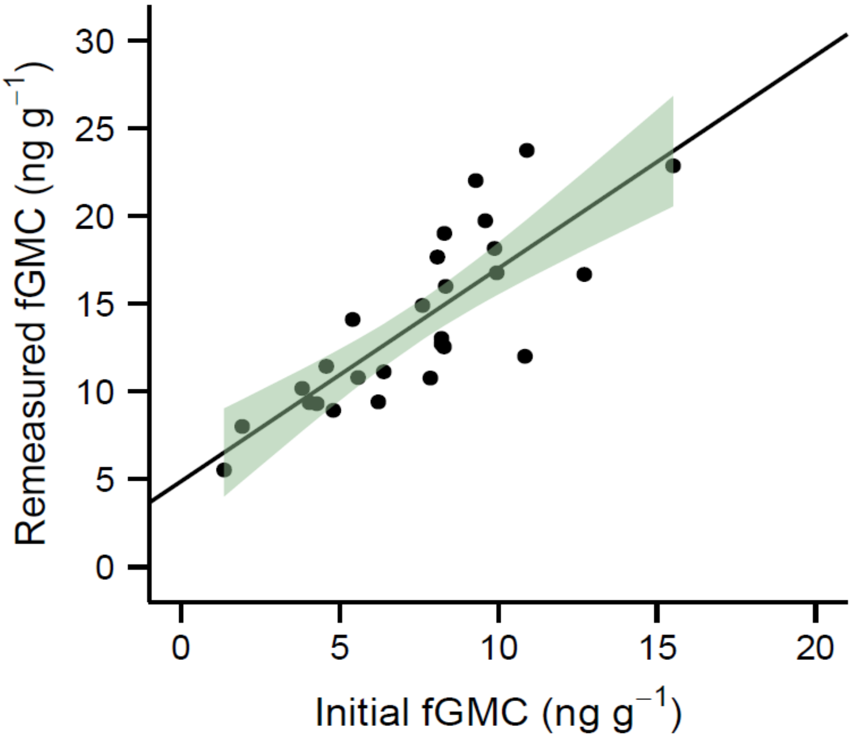

Fig. 4. Relationship between initial and remeasured faecal glucocorticoid metabolite concentrations (fGMC) for a subset of 27 faecal samples. The black line is the OLS linear regression fitted to predict fGMCs in Cluster 2 (eqn 2: $fGMC_{remeasured} = 4.88 + 1.21 \times fGMC_{initial}$, adjusted $r^2 = 0.65$). The area shaded in green represents the 95% confidence interval of the fit.

Applying and validating the standardization procedure with a subset of 27 samples

To test the effectiveness of the standardization based on a subset of samples, we randomly chose 27 samples from our complete data set of 138 samples and fitted an OLS linear regression to their initial and remeasured fGMCs (adjusted $r^2 = 0.65$; n = 27; Fig. 4). The resulting equation was as follows:

$$fGMC_{remeasured} = 4.88 + 1.21 \times fGMC_{initial} \qquad \text{eqn 2}$$

The cross-validation of the model indicated that 88.9% of samples (24 out of 27 samples) had a CVfit ≤20%, confirming that our subset was large enough to perform reliable standardization. Moreover, the Pearson product–moment correlation coefficient between predicted and remeasured fGMCs for the model based on the subset of samples was high (eqn 2, r = 0.81, n = 27) and did not differ from the correlation obtained for the model based on the complete data set (eqn 1, r = 0.85, n = 138; Fisher's r to Z transformation; Z = 0.58, P = 0.56).

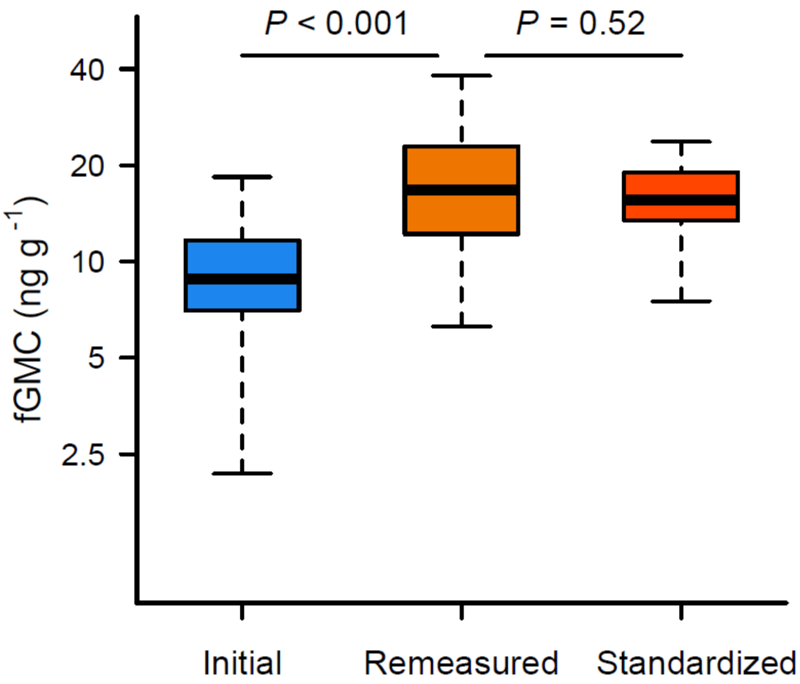

Fig. 5. Faecal glucocorticoid metabolite concentrations (fGMC) initially measured in Cluster 1, remeasured with the accuracy of Cluster 2 and standardized on Cluster 2 (n = 138). Boxes encompass interquartile ranges (first to third quartiles around the median), horizontal lines inside boxes represent medians, and whiskers are at 1.5 times the interquartile ranges. The y-axis is displayed on a logarithmic scale.

We standardized the fGMCs of all 138 samples of Cluster 1 using eqn 2 and rescaled the raw standardized concentrations into final concentrations by multiplying them by their dilution factor. To test the effect of the variation in EIA accuracy on the quantification of fGMCs, assess the potential risk of misinterpreting results when fGMCs of different accuracy are combined, and verify that our standardization procedure effectively reduced such risk, we compared the fGMCs of the 138 samples that were remeasured with the accuracy of Cluster 2 to (i) their matched fGMCs initially measured in Cluster 1 and (ii) their matched fGMCs after standardization on Cluster 2.As expected by the observed increase in pool concentrations, the fGMCs remeasured with the accuracy of Cluster 2 (median = 16.7 ng g^{-1}) were significantly higher than their matched fGMCs measured in Cluster 1 (median = 8.8 ng g^{-1}; Wilcoxon's signed-rank test, V = 9591, $P < 0.0001$; median of between-group differences = 7.44 ng g^{-1}, CI95% = 6.76–8.33 ng g^{-1}; Fig. 5), but did not significantly differ from their matched standardized fGMCs (median = 15.6 ng g 1; V = 4494, *P* = 0.52; median of between-group differences = 0.30 ng g^{-1}, CI95% = 0.84 to 0.42 ng g^{-1}; Fig. 5).

DISCUSSION

Our results confirm that changes in EIA accuracy can bias measurements of metabolite concentrations. Measurements of varying accuracy should therefore be standardized before being combined for statistical analysis. We showed that our method reduced the differences in fGMCs caused by the change in EIA accuracy between two clusters to a non-significant value, rendering all measurements of the study comparable with each other. We further demonstrate the reliability of the standardization procedure when only a small subset of samples is re-assayed. To our knowledge, this is the first method to standardize metabolite concentrations when changes in accuracy occur within a given EIA.

The method involves simple statistical procedures, applies relevant and widely accepted criteria in endocrinology and can be generalized to cases when two or more clusters require standardization (see Box 1 for a summary of the procedure). Appropriate consideration should be given to the number of samples to re-assay and the regression method applied to model the relationship between initial and remeasured metabolite concentrations. The minimum number of samples to reassay is study specific and depends on various factors such as the maximum intra-assay and interassay variation in precision and accuracy that is tolerated (here, 5% and 20%, respectively), the threshold for model acceptance and the dispersion of sample metabolite concentrations (Linnet 1999; Brown, Walker & Steinman 2004). Here, we proposed a threshold for model acceptance of 70%of samples complying with our criterion of prediction reliability, and this indicated a minimum subset of 27 samples to be re-assayed. The model fitted to 27 samples was as effective at standardizing fGMCs as the model for the complete data set, confirming that this threshold was sufficient in our case. A different threshold may be better suited to other data sets and scientific questions. Note, however, that even when fitted to the complete data set, the mean predictive performance of our model never exceeded 87. 5% (see Table S2, Appendix S1 in Supporting Information). We suggest starting with a subset of approximately 27 samples and cross-validating the fitted model to assess a posteriori whether predictions are reliable or whether additional samples need to be re-assayed. The approximate number of additional samples required to reach the chosen threshold can be estimated with the help of

Table S2 (Appendix S1 in Supporting Information) for two different criteria of prediction reliability.

Whether the samples in the subset should be chosen in a random manner from the complete data set or randomly within some stratification may depend on the scientific question and the distribution of metabolite concentrations in the cluster. If measurements reflect different treatments or categories of individuals (e.g. diet, age, sex, social status), samples should be randomly chosen within each treatment or category of individuals (Pocock & Simon 1975). To avoid introducing errors owing to different dilution factors, we further recommend fitting a model on measured raw metabolite concentrations, that is, not corrected for their dilution. The chosen subset should therefore be restricted to samples that can be re-assayed at the same dilution as their initial measurement. The factor of change in concentration of the high and low pools between two clusters can be used to estimate the highest and lowest initial raw metabolite concentration in samples that is likely to fall within the linear range of the calibration curve if remeasured at the same dilution.

The relationship between initial and remeasured concentrations may in most cases be best described by a linear regression, but other methods (e.g. polynomial) may give a better fit depending on how concentrations changed along with the change in EIA accuracy. Alternatively, nonparametric regression techniques such as splines (Green & Silverman 1993) may be applied. In our study, we only accepted measurements when their duplicated values differed by <5%. Applying a simple OLS linear regression was as effective at standardizing metabolite concentrations as a more complex model that explicitly incorporated measurement errors (see Appendix S1 in Supporting Information). If a larger discrepancy between duplicated measurements is tolerated, OLS linear models may have a lower predictive power than models that incorporate measurement errors on both axes.

The standardization relies on the ability to track changes in EIA accuracy using the metabolite concentration of pools. It is thus important to be able to dismiss the effect of erroneous preparation of hormone standard and pool solutions on metabolite measurements. To facilitate this, we highly recommend preparing new stock solutions of pools and standards before the old stocks are depleted and assaying them together on a transition plate to ensure that new solutions give similar results to the old ones (Brown, Walker & Steinman 2004). When old and new stock solutions of pools are prepared based on different faecal or urinary samples, for example when the original samples are depleted, such a procedure allows to adjust the dilution of new solutions to match the concentration of old pools or, alternatively, to calculate a factor of change between old and new pools. The evaluation of the performance of our EIA indicated that the EIA remained highly precise and sensitive throughout the study. The validation of parallelism at the beginning and end of the study confirmed the absence of matrix effects that could have been associated with the extraction procedure, denaturation of the antibody or changes in the structure of metabolites in faecal extracts over time. Renewal of stock solutions of standards and faecal pools did not coincide with changes in the optical densities of standards or with changes in the metabolite concentration of pools. Finally, the switch in laboratory personnel that occurred between the two clusters is unlikely to be the cause of the change in EIA accuracy because several technicians used the same EIA during our study and all experienced a similar change in binding sensitivity and accuracy. The observed change in accuracy and binding sensitivity between the two clusters thus most probably resulted from adjustments in the concentration of antibody and tracer and potential (uncontrolled) fluctuations in environmental conditions (e.g. room temperature).

Our assessment of the standardization procedure was based on re-assaying a subset of samples after some time had elapsed (18 months and more). Ageing of faecal samples, that is, the latencies between sample collection, storage, extraction and assaying, may alter metabolite concentration owing to naturally occurring faecal bacteria that may decompose steroid metabolites after defaecation (Möstl & Palme 2002). Applying appropriate treatment, storage and extraction procedures can stabilise hormone metabolites for long periods of time, possibly for many years. Here, we applied the recommended treatment and storage procedures for faecal steroids (Khan et al. 2002; Terio et al. 2002; Hunt & Wasser 2003; Lynch et al. 2003; Millspaugh&Washburn 2004;Kalbitzer&Heistermann 2013) and found that the increase in fGMC of sample extracts was consistent with that of pools, suggesting that the age of extracts had no or only a minor influence on the measurements.

Quantifying the absolute accuracy of metabolite measurements is difficult because the chemical structure and binding affinity of metabolites are usually unknown. We therefore cannot determine whether the initial or the remeasured metabolite concentrations are more accurate. This is usually of little relevance in studies in ecology and evolution where a similar level of EIA accuracy and comparable measurements are more important than a high EIA accuracy in absolute terms (Lynch et al. 2003). Methods have been developed within the context of clinical studies and studies in conservation medicine to compare and harmonise measurements of plasma hormones obtained with different EIAs (Bidlingmaier & Freda 2010). However, the global application of such harmonisation methods may be limited because they are often based on the systematic re-assaying of complete sets of samples, are not aimed at predicting standardized concentrations and rarely consider intra-EIA changes in accuracy, sensitivity or precision that would affect the harmonisation procedure over time (Müller et al. 2011).

Our standardization method may be particularly useful for collaborative projects that share the laboratory workload between different facilities and are likely to experience variation in EIA performance and accuracy, and for long-term and longitudinal studies that typically deal with large data sets and may not be able to re-assay all samples whenever new samples are collected or a new research question is investigated. Moreover, because this method only requires the re-assaying of a subset of samples, it allows the standardization of the initial measurements of samples that are no longer available. This can significantly increase sample sizes, enhance the power of statistical analyses and allow the inclusion of a larger number of covariates in statistical models, which may be important for a better understanding of complex processes.

Box 1. Procedure to standardize sample metabolite concentrations measured by EIAs.

Step 1: Identify clusters of samples assayed with similar EIA accuracy

- Define the EIA accuracy at the time of standardisation as the reference accuracy. If unknown, run a plate with standards and pools to determine it.
- Conduct a hierarchical cluster analysis on the concentration of pools of all plates and assign clusters based on the resulting dendrogram.
- Calculate the inter-assay coefficient of variation (CV) of pools for each cluster; subdivide clusters with CVs exceeding the criterion for similar EIA accuracy (here, 20%). Repeat until all clusters have CVs that satisfy the criterion.
- Define the cluster of samples assayed with the reference accuracy as the reference cluster and standardise samples from all other clusters on this cluster.

Note: The following steps describe the procedure to standardise one cluster. If Step 1 indicates that more clusters should be standardised, repeat Step 2 to Step 5 for each cluster.

Step 2: Choose and re-assay a subset of samples

- Choose a subset of samples that is representative of all samples of the cluster and that can be re-assayed at the same dilution as when initially assayed.
- Re-assay the subset within a few days after the cluster analysis to ensure that the EIA accuracy of the subset and reference cluster are similar.

Step 3: Model the relationship between initial and re-measured metabolite concentrations

- Model the relationship between initial (x) and re-measured (y) concentrations of the subset of samples using raw measurements, i.e. before multiplying them by their dilution factor, and retrieve the resulting equation (i.e. intercept and slope).

Step 4: Test the predictive performance of the model

- Cross-validate the model and retrieve the predicted metabolite concentrations of the samples in the subset.
- Set a criterion for prediction reliability that corresponds to the criterion for similar EIA accuracy (here, 20%) and compute the CVs of the predicted and re-measured metabolite concentrations for each sample in the subset.

● Set a threshold for satisfying model predictive performance (here, 70%) and calculate the percentage of samples that conform to the criterion for prediction reliability. If this threshold is not reached, re-assay more samples (i.e. restart from Step 2).

Step 5: Standardise the metabolite concentrations

● Standardise the concentrations of all other samples of the cluster using the equation obtained in Step 3.

● Rescale the standardised raw concentrations into final concentrations by multiplying them by their dilution factor.

Note: R programing codes for each step of the standardisation procedure are provided as Supporting Information in Appendix S2.

ETHICAL STATEMENT

All procedures were performed in accordance with the requirements of the Leibniz Institute for Zoo and Wildlife Research Ethics Committee on Animal Welfare.

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DATA ACCESSIBILITY

The data are archived in figshare: http://dx.doi.org/10.6084/m9.figshare.1298131.

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SUPPORTING INFORMATION (APPENDIX S1)

This document contains:

1. EIA performance in each batch of measurements (Table S1)
2. Alternative regression method that considers measurement errors
3. Variation in model predictive performance with decreasing size of data set (Table S2)

1. EIA performance in each batch of measurements

To assess the performance of our enzyme immunoassay (EIA) during each of the three batches of measurements (July 2011, September 2011 and July 2013), we quantified the following parameters:

Precision: refers to the repeatability of measurements of metabolite concentrations of (faecal or urinary) control pools and is divided into two components: (1) the intra-assay precision which corresponds to the within-plate difference in duplicated measurements of pools, and (2) the interassay precision which corresponds to the between-plate difference in repeated measurements of pools. Intra- and interassay precision are traditionally expressed as the coefficient of variation (CV) of repeated measurements. As a rule of thumb, results were considered precise when the $CV_{intra\text{-}assay}$ and $CV_{interassay}$ did not exceed 5% and 20%, respectively. In concordance with the criterion for high interassay precision, EIA accuracy was considered stable across plates when $CV_{interassay}$ did not exceed 20%. The formula of the CV that was applied to calculate the $CV_{intra\text{-}assay}$, CVinterassay and CV_{fit} (see main document) was:

$$CV = SD / mean \times 100$$

where *SD* and *mean* correspond to the standard deviation and the mean of a given set of measurements, respectively. Note that we computed all SDs using the denominator (n-1), with *n* being the number of measurements considered. Results are presented in Table S1.

Analytical sensitivity (S): refers to the ability of an EIA to respond, in terms of optical densities (ODs), to small variations in concentration (i.e. ‘slope’ definition of sensitivity, Pardue 1997). It corresponds to the slope Si of the tangent at a given point i of the calibration curve as follows:

$$Si = \Delta OD[i,i+h] / \Delta Conc[i,i+h]$$

where $\Delta OD[i,i+h]$ is the difference in OD between two points within a small interval $[i,i+h]$ and $\Delta Conc[i,i+h]$ is the difference between the corresponding concentrations of the two points. We used the mean calibration curve for each batch of analyses to calculate the EIA sensitivity and quantitative resolution (see below) for three standard values (1.56 ng/g; 6.25 ng/g; 25 ng/g; Table S1).

Quantitative resolution (QR): although it is rarely considered, the QR is a useful parameter to evaluate the quality of the estimation of concentrations at a given point i of the calibration curve and is calculated as follows:

$$QRi = SD(OD)i / Si$$

where $SD(OD)i$ is the standard deviation of the ODs of standard duplicates of a point i and Si is the analytical sensitivity (Pardue 1997) at i. Small values of quantitative resolution indicate a fine-tuned quantification of sample concentrations (Table S1).

Table S1: EIA performance in terms of precision, stability of EIA accuracy, analytical sensitivity and quantitative resolution in July 2011, September 2011 and July 2013. Stability of EIA accuracy and intra- and interassay precision were quantified through the coefficient of variation (CV) of repeated measurements of faecal pools with relatively low ('low pool') and high ('high pool') metabolite concentration. 'n' refers to the number of plates run within each batch of analyses.

		Jul-11 **(n = 5)**	**Sep-11** **(n = 5)**	**Jul-13** **(n = 13)**
$CV_{intra\text{-}assay}$ (%)	low pool	2	3	2
	high pool	2	2	1
$CV_{interassay}$ (%)	low pool	8	20	15
	high pool	15	3	8
Analytical sensitivity	1.56 ng/g	0.049	0.137	0.11
	6.25 ng/g	0.012	0.021	0.059
	25 ng/g	0.001	0.003	0.005
Quantitative resolution	1.56 ng/g	0.17	0.08	0.16
	6.25 ng/g	0.46	0.19	0.09
	25 ng/g	1.13	0.59	1.31

2. Alternative regression method that considers measurement errors

Directly applying an ordinary least-squares (OLS) linear regression to model the relationship between repeated measurements of concentrations typically violates the assumption of exogeneity because the independent variable (here: fGMCinitial) was measured with error. This may lead to biased regression estimates. Here, we present a linear regression method that takes into account these measurement errors when fitting a model, and compare its resulting regression coefficients and predictive power to that of the simple OLS linear regression applied in the main document.

Repeated ordinary least-squares (OLS) linear regressions on simulated values of x and y: The measurement error of the concentration of a given sample can be estimated using the standard error of sample duplicates. To consider this source of variation in our model, we simulated data by randomly drawing a value of x ($fGMC_{initial}$) and y ($fGMC_{remeasured}$) for each sample from a normal distribution based on the corresponding mean and standard error of sample duplicates and fitted an OLS linear regression. We repeated the simulation and fitting procedures one thousand times and used the means of the regression coefficients (i.e. mean slope and mean intercept) generated from the 1000 OLS linear regressions to determine a unique equation to predict fGMCs in Cluster 2 (mean adjusted $r^2 = 0.73$; $n = 138$):

$$fGMC_{remeasured} = 4.29 + 1.32 \times fGMC_{initial} \qquad \text{(eqn S1)}$$

The predictive power (i.e. adjusted r^2) and mean regression coefficients of this model were very similar (ANOVA, comparison of the regression slopes; $F = 0.01$, d.f. = 2, $P = 0.99$) to that of the OLS linear regression presented in the main document (adjusted $r^2 = 0.72$; $n = 138$):

$$fGMC_{remeasured} = 4.22 + 1.33 \times fGMC_{initial} \qquad \text{(eqn S2)}$$

This indicates that considering measurement errors in our model did not significantly improve the reliability of the model predictions. We thus only presented the simplest linear regression approach in the main document.

Note that we considered using ‘error-in-variables’ models such as a Major Axis linear regression and other similar methods such as the weighted Deming linear regression and the Maximum-Likelihood regression (MLFR) which are recommended to describe the functional relationship between two variables when both the dependent and independent variables are associated with measurements errors. In contrast to OLS linear regressions, such models fit an equation that minimises the sum of squared Euclidean distances between each data point and the regression line (Legendre & Legendre 1998). Such models are commonly used to compare results from different immunoassays (e.g. Cornbleet & Gochman 1979, Ripley & Thompson 1987; Linnet 1990; Wild 2013). ‘Error-in-variables’ models are particularly useful to make inferences on a model’s parameters. However, their application within a predictive context has been questioned because of their potential to create biased predictions (e.g. Sokal & Rohlf 1987; Legendre & Legendre 1998; Smith 2009). To avoid this risk we chose to present the results from the OLS linear regression in the main document.

3. Variation in model predictive performance with decreasing size of data set

Table S2: Predictive performance of ordinary least squares (OLS) linear regressions fitted on data sets of decreasing size (from n = 138 to n = 10 faecal samples). The random selection of faecal samples and OLS fitting procedures were reiterated 10 000 times for each data set. Results indicate the mean, upper and lower confidence limits at 95% of the percentage of faecal samples in each data set for which the coefficient of variation (CVfit) between predicted and remeasured metabolite concentrations did not exceed 20% and 10%.

	CV_{fit} ≤ 20 %			CV_{fit} ≤ 10 %		
Size of data set	**mean**	**upper $CI_{95\%}$**	**lower $CI_{95\%}$**	**mean**	**upper $CI_{95\%}$**	**lower $CI_{95\%}$**
138	87.5	89.1	86.2	51.3	54.4	47.8
137	87.5	89.1	86.1	51.4	54.7	48.2
136	87.5	89.0	86.0	51.3	54.4	47.8
135	87.5	88.9	85.9	51.3	54.8	48.1
134	87.5	89.6	85.8	51.3	54.5	47.8
133	87.5	89.5	85.7	51.3	54.9	47.4
132	87.5	89.4	85.6	51.4	55.3	47.7
131	87.5	89.3	85.5	51.4	55.0	47.3
130	87.5	90.0	85.4	51.3	55.4	46.9
129	87.5	89.9	85.3	51.4	55.0	47.3
128	87.5	89.8	85.2	51.3	55.5	47.7
127	87.5	89.8	85.0	51.4	55.1	47.2
126	87.5	89.7	84.9	51.3	55.6	46.8
125	87.5	90.4	84.8	51.3	56.0	47.2
124	87.5	90.3	84.7	51.3	55.7	46.8
123	87.5	90.2	85.4	51.3	56.1	47.2
122	87.5	90.2	85.3	51.4	55.7	46.7
121	87.5	90.1	85.1	51.4	56.2	46.3
120	87.4	90.0	85.0	51.4	55.8	46.7
119	87.5	90.8	84.9	51.4	56.3	46.2
118	87.5	90.7	84.8	51.3	55.9	46.6
117	87.5	90.6	84.6	51.4	56.4	46.2
116	87.5	90.5	84.5	51.4	56.0	46.6
115	87.5	90.4	84.4	51.4	56.5	46.1
114	87.4	90.4	84.2	51.4	56.1	46.5
113	87.5	91.2	84.1	51.4	56.6	46.0
112	87.5	91.1	83.9	51.4	56.3	46.4
111	87.4	91.0	83.8	51.4	56.8	46.0
110	87.5	90.9	83.6	51.3	57.3	45.5
109	87.5	90.8	84.4	51.4	56.9	45.9
108	87.4	90.7	84.3	51.4	57.4	45.4
107	87.4	90.7	84.1	51.4	57.0	45.8

106	87.5	91.5	84.0	51.3	57.6	45.3
105	87.5	91.4	83.8	51.4	57.1	45.7
104	87.5	91.4	83.7	51.5	57.7	45.2
103	87.4	91.3	83.5	51.4	57.3	45.6
102	87.4	91.2	83.3	51.4	57.8	45.1
101	87.4	91.1	83.2	51.4	57.4	45.5
100	87.4	92.0	83.0	51.4	58.0	45.0
99	87.4	91.9	82.8	51.4	57.6	45.5
98	87.4	91.8	83.7	51.4	58.2	44.9
97	87.4	91.8	83.5	51.5	57.8	44.3
96	87.4	91.7	83.3	51.4	58.3	44.8
95	87.4	91.6	83.2	51.4	57.9	44.2
94	87.3	91.5	83.0	51.4	58.5	44.7
93	87.4	91.4	82.8	51.5	58.1	44.1
92	87.4	92.4	82.6	51.4	58.7	44.6
91	87.4	92.3	82.4	51.5	59.3	44.0
90	87.4	92.2	82.2	51.4	58.9	44.4
89	87.4	92.1	82.0	51.5	58.4	43.8
88	87.3	92.1	81.8	51.4	59.1	43.2
87	87.4	92.0	82.8	51.5	58.6	43.7
86	87.3	91.9	82.6	51.5	59.3	44.2
85	87.3	91.8	82.4	51.5	58.8	43.5
84	87.3	92.9	82.1	51.5	59.5	44.1
83	87.3	92.8	81.9	51.5	59.0	43.4
82	87.3	92.7	81.7	51.5	59.8	43.9
81	87.3	92.6	81.5	51.5	59.3	43.2
80	87.3	92.5	81.3	51.6	60.0	43.8
79	87.3	92.4	81.0	51.6	59.5	43.0
78	87.2	92.3	82.1	51.5	60.3	43.6
77	87.2	92.2	81.8	51.5	59.7	42.9
76	87.2	93.4	81.6	51.5	60.5	43.4
75	87.2	93.3	81.3	51.6	60.0	42.7
74	87.2	93.2	81.1	51.6	60.8	41.9
73	87.2	93.2	80.8	51.5	60.3	42.5
72	87.1	93.1	80.6	51.5	61.1	41.7
71	87.1	93.0	80.3	51.5	60.6	42.3
70	87.2	92.9	81.4	51.5	61.4	41.4
69	87.0	92.8	79.7	51.7	60.9	42.0
68	87.1	92.7	80.9	51.6	61.8	42.7
67	87.1	92.5	80.6	51.6	61.2	41.8
66	87.0	93.9	80.3	51.7	62.1	40.9
65	87.0	93.9	80.0	51.7	61.5	41.5
64	87.0	93.8	79.7	51.6	62.5	40.6
63	87.0	93.7	79.4	51.7	61.9	41.3
62	87.0	93.6	79.0	51.6	62.9	40.3

61	86.9	93.4	78.7	51.6	62.3	41.0
60	86.9	93.3	80.0	51.7	61.7	41.7
59	86.8	93.2	79.7	51.7	62.7	40.7
58	86.8	93.1	79.3	51.6	62.1	41.4
57	86.8	94.7	79.0	51.6	63.2	40.4
56	86.8	94.6	78.6	51.7	62.5	39.3
55	86.8	94.6	78.2	51.6	63.6	40.0
54	86.8	94.4	77.8	51.7	63.0	40.7
53	86.6	94.3	77.4	51.8	64.2	39.6
52	86.6	94.2	78.9	51.7	63.5	40.4
51	86.6	94.1	78.4	51.7	64.7	39.2
50	86.5	94.0	78.0	51.8	64.0	40.0
49	86.6	93.9	77.6	51.8	63.3	38.8
48	86.5	95.8	77.1	51.7	64.6	39.6
47	86.5	95.7	76.6	51.8	63.8	38.3
46	86.4	95.7	76.1	51.8	65.2	39.1
45	86.4	95.6	75.6	51.7	64.4	37.8
44	86.3	95.5	75.0	51.6	65.9	38.6
43	86.2	95.4	76.7	51.6	65.1	37.2
42	86.2	95.2	76.2	51.8	66.7	38.1
41	86.1	95.1	75.6	51.7	65.9	36.6
40	86.1	95.0	75.0	51.6	67.5	37.5
39	86.0	94.9	74.4	51.7	66.7	35.9
38	85.8	94.7	73.7	51.7	65.8	36.8
37	85.9	94.6	73.0	51.8	67.6	35.1
36	85.8	94.4	75.0	51.5	66.7	36.1
35	85.7	97.1	74.3	51.6	68.6	34.3
34	85.6	97.1	73.5	51.7	67.7	35.3
33	85.6	97.0	72.7	51.6	66.7	36.4
32	85.5	96.9	71.9	51.6	68.8	34.4
31	85.3	96.8	71.0	51.6	67.7	35.5
30	85.2	96.7	70.0	51.5	70.0	33.3
29	85.2	96.6	72.4	51.4	69.0	34.5
28	85.0	96.4	71.4	51.3	71.4	32.1
27	84.9	96.3	70.4	51.3	70.4	33.3
26	84.8	96.2	69.2	51.3	69.2	30.8
25	84.6	96.0	68.0	50.9	72.0	32.0
24	84.5	95.8	66.7	51.1	70.8	29.2
23	84.2	95.7	65.2	51.2	73.9	30.4
22	84.1	100.0	68.2	51.2	72.7	31.8
21	83.9	100.0	66.7	51.1	71.4	28.6
20	83.8	100.0	65.0	50.9	75.0	30.0
19	83.5	100.0	63.2	50.6	73.7	26.3
18	83.1	100.0	61.1	50.3	72.2	27.8
17	82.9	100.0	58.8	50.2	76.5	23.5

16	82.6	100.0	62.5	50.1	75.0	25.0
15	82.2	100.0	60.0	49.5	73.3	20.0
14	82.0	100.0	57.1	49.3	78.6	21.4
13	81.3	100.0	53.9	48.9	76.9	23.1
12	80.9	100.0	50.0	48.7	75.0	16.7
11	80.1	100.0	45.5	48.4	81.8	18.2
10	79.1	100.0	50.0	47.3	80.0	10.0

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SUPPORTING INFORMATION (APPENDIX S2)

This document contains the *R* software programming codes to standardise sample metabolite concentrations when the accuracy of an enzyme immunoassay (EIA) varies. The procedure, divided in 5 Steps, is described in the main document (Methods & Results section) and summarised in Box 1. These codes can be run using our exemplary data sets, archived as text files on figshare.

########################## Standardisation procedure ############################

 # Step 1: Identify clusters of samples assayed with similar EIA accuracy

```
data.cluster <- read.table(file="data_for_cluster.txt", header=TRUE) # Imports the file containing the concentrations of low pool ("Low.Pool") and high pool ("High.Pool") assayed on each plate(here, n = 23).

head(data.cluster) # Displays the first 6 rows of data.cluster (to check that the file was imported properly).

dissimilarity.matrix <- dist(x=data.cluster, method="euclidean") # Creates a dissimilarity matrix based on Euclidean distances.

hierarchical.clustering <- hclust(d=dissimilarity.matrix, method="ward.D2") # Performs a hierarchical clustering.

plot(hierarchical.clustering) # Plots the resulting dendrogram.
```

 # Step 2: Choose and re-assay a subset of samples

```
# If a subset should be chosen randomly from the complete data set, the following codes can be applied:

Ntotal <- 138 # Sets the total number of samples (here 138).

Nsubset <- 27 # Sets the size of the subset (here 27).

subset <- sort(x=sample(x=1:Ntotal)[1:Nsubset]) # Choses a random subset of size Nsubset from the entire data set of size Ntotal. Note that the result will always be different as it is random.

subset # Displays the randomly chosen samples to re-assay.
```

 # Step 3: Model the relationship between initial and remeasured metabolite concentrations

```
data.subset <- read.table(file="data_for_fit.txt", header=TRUE) # Imports the file containing the initial raw concentrations in Cluster 1 ("x.raw") and the remeasured raw concentrations in Cluster 2 ("y.raw") for the chosen subset (here 27 samples).

head(data.subset) # Displays the first 6 rows of data.subset (to check that the file was imported properly).

 fit.subset <- lm(formula=y.raw~x.raw, data=data.subset) # Fits an OLS linear regression on the subset.

summary(fit.subset) # Displays model equation and adjusted r squared.
```

 # Step 4: Test the predictive performance of the model

```
install.packages("DAAG") # Installs DAAG package.

library(DAAG) # Loads DAAG package.

CV <- function(x) 100*sd(x=x)/mean(x=x) # Defines the function to compute the coefficient of variation.

CVfit <- function(CVlm) apply(cbind(CVlm$cvpred, CVlm$y.raw), 1, CV) # Defines the function to compute "CVfit", i.e. the coefficient of variation between predicted ("CVlm$cvpred") and remeasured concentrations ("CVlm$y.raw"), for each sample in the subset. Note that the "CV" in the name of the function (CVlm) from the DAAG package stands for Cross-Validation and not for coefficient of variation.

CrossValidation <- CVlm(df=data.subset, form.lm=y.raw~x.raw) # Performs the cross-validation on the subset.

CVfit.values <- CVfit(CVlm=CrossValidation) # Runs the CVfit function as defined above on the cross-validation results.

CVfit.values # Displays the coefficient of variation between predicted and remeasured concentrations for each sample in the subset.

criterion <- 20 # Sets the cut-off value for coefficients of variation (here 20%).
```

Predictive_performance <- mean(x=CVfit.values <= criterion)*100 # Calculates the model's predictive performance, i.e. the percentage of samples for which CVfit ≤ criterion.

Predictive_performance # Displays the resulting model's predictive performance.

 # Step 5: Standardise the metabolite concentrations

data.std <- read.table(file="data_for_standardisation.txt", header=TRUE) # Imports the file containing all 138 initial raw concentrations measured in Cluster 1 ("x.raw"), the remeasured raw concentrations in Cluster 2 ("y.raw"),the associated dilution factor ("dilution") and corresponding final concentrations in Cluster 1 ("x.final") and Cluster 2 ("y.final").The last column to the right indicates whether or not the sample was part of the chosen subset of 27 samples to fit the model.

head(data.std) # Displays the first 6 rows of data.std (to check that the file was imported properly).

data.std$y.raw.standardised <- predict(object=fit.subset, newdata=list(x.raw=data.std$x.raw)) # Computes the predicted (i.e. standardised) raw concentrations in Cluster 2 ("y.raw.standardised") using the OLS linear regression equation fitted on the subset, for all samples from Cluster 1 ("data.std$x.raw").

data.std$y.final.standardised <- data.std$y.raw.standardised*data.std$dilution # Calculates the final standardised concentrations in Cluster 2 (in ng/g) by multiplying the standardised concentrations ("data.std$y.raw.standardised") by their dilution factor in Cluster 1 ("data.std$dilution").

data.std$y.final.standardised # Displays the final table.

CHAPTER 5

Physiological stress as a mediator of rank-related reproductive investment and reproductive skew in a social mammal

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The conceptual framework and predictions of this study were developed by Eve Davidian (ED) and Oliver Höner (OH). Demographic monitoring and collection of behavioral data and faecal samples were done by ED, OH, and Bettina Wachter (BW). Ilja Heckmann (IH) developed the program in Python software to extract data on the social rank and on hourly sightings and proximity of individual hyenas. The cortisol-3-CMO enzyme immunoassay was previously developed and validated for spotted hyenas by Martin Dehnhard (MD), Sarah Benhaiem, Marion East and Heribert Hofer (HH). Faecal sample freeze-drying, metabolite extraction, and measurements of concentrations in cortisol metabolites were conducted by technical assistants (Heidrun Barleben, Sylvia Schultz van Endert and Katrin Paschmionka) under the supervision of ED and MD. Colin Vullioud conducted the Bayesian model, using the institute's computer cluster, under the guidance of ED. ED conducted all other statistical analyses, under the guidance of OH and HH. The manuscript was primarily written by ED and OH, and was reviewed by BW, IH MD, and HH.

Abstract

In many animal societies, socially dominant males sire more offspring and/or offspring of higher quality than subordinate males. The proximate mechanisms by which social rank influences reproductive success are poorly understood. Here, we investigated the influence of rank-related dominance potential and socially-induced physiological stress on male sociality and courtship behaviour in free-ranging spotted hyenas (*Crocuta crocuta*). Physiological stress was similar in all males when they courted females and competitors were absent or when they were resting alone. In contrast, when males engaged in social activities or courted females when competitors were present, low-ranking males, who have a low dominance potential, had higher physiological stress than high-ranking males. Low-ranking males adjusted their behaviour to their stronger physiological constraints by minimising intrasexual competition; they spent more time alone, less time engaging in social and sexual activities, and they invested less in the most attractive and most contested females than did high-ranking males. These behavioural adjustments allowed low-ranking males to downregulate their physiological stress but also reduced their chances to be chosen as sire, explaining why their reproductive success is both quantitatively and qualitatively lower than that of high-ranking males. Our results demonstrate that male dominance potential mediates the physiological costs of intrasexual competition and shapes behavioural trade-offs between the allocation of time and physiological resources to social integration, reproduction, and self-maintenance. Our study shows that physiological constraints can play a pivotal role in the emergence of rank-related male reproductive investment and reproductive skew in group-living species.

Keywords: Physiological constraints; Social status; Performance; Faecal glucocorticoids; Stress coping outlets; Reproductive tactics; Activity budget; Trade-off; Natal dispersal

Introduction

Understanding the causes and consequences of inter-individual variation in reproductive success is one of the main goals of studies in behavioural ecology and evolutionary biology. In most group-living species in which societies are structured by social hierarchies, social rank is a primary determinant of reproductive success, with individuals of high rank typically producing more offspring and/or offspring of higher quality than those of lower rank (Cowlishaw and Dunbar, 1991; Ellis, 1995; Keller and Reeve, 1994). Extensive effort has been made to investigate how natural and sexual selection act on rank-related traits (Andersson, 1994; Clutton-Brock, 2007; Clutton-Brock and Huchard, 2013) and to identify the causes of variation in reproductive skew within and between species (Alberts, 2012; Ellis, 1995; Gogarten and Koenig, 2012; Keller and Reeve, 1994; van Noordwijk and van Schaik, 2004). Yet the proximate mechanism by which social rank influences reproductive success remains largely unknown (Cavigelli and Caruso, 2015; Dantzer et al., 2016; Moore and Hopkins, 2009).

Sexual selection theory predicts that, in males, reproductive success is primarily determined by the number of receptive females and male reproductive performance in terms of their ability to access mates and create breeding opportunities (Andersson, 1994). In multimale, multifemale societies, top-ranking males are predicted to have a competitive advantage over subordinate males, benefit from preferential access to mating partners, and sire the largest number of offspring of their group (Alberts et al., 2003; Ellis, 1995; Kutsukake and Nunn, 2009; Port and Cant, 2014; Reeve et al., 1998). However, dominant males rarely monopolise reproduction and their reproductive investment and success usually decreases when the number of male competitors increases (Alberts, 2012; Alberts et al., 2003; Cowlishaw and Dunbar, 1991; Gogarten and Koenig, 2012; Setchell et al., 2005). This suggests that the intensity of intrasexual competition plays an important role in shaping rank-related performance (Cowlishaw and Dunbar, 1991; Gogarten and Koenig, 2012). It also suggests that a male’s reproductive performance may be determined by his density-dependent ability to cope with the costs of competition, rather than his intrinsic or social attributes (e.g., body size, aggressiveness, sperm quality, age or number of coalitionary partners) which may be associated with his social rank.

Intrasexual competition over acquisition and maintenance of social rank and over access to mates can cause costly changes in physiological traits such as the concentration of glucocorticoids (Creel et al., 2013; Goymann and Wingfield, 2004), termed hereafter, ‘physiological stress’. Glucocorticoids are key physiological mediators of the endocrine control mechanisms that regulate essential biological functions (McEwen and Wingfield, 2003; Romero et al., 2009), and influence individual life history and fitness (Dantzer et al., 2016; Ricklefs and Wikelski, 2002). Whereas an acute increase in physiological stress usually constitutes an adaptive physiological response to a brief challenge, maintaining elevated physiological stress as a result of sustained exposure to challenges can disrupt an individual’s endocrine control mechanism, its ability to respond adequately to subsequent challenges, and impair its fitness (DuRant et al., 2016; MacLeod et al., 2018; McEwen and Wingfield, 2003; Romero et al., 2009). Males occupying a rank within the top third of the hierarchy (hereafter, ‘high-ranking’ males) and males occupying a rank within the lowest third (hereafter, ‘low-ranking’ males) usually differ in their exposure to and ability to cope with social conflicts (hereafter, ‘dominance potential’). High-ranking males typically are less likely to lose an agonistic interaction or be the target of dominance acts, have relatively more targets they can dominate, and have more coalition partners and stronger social bonds than low-ranking males (Cavigelli and Caruso, 2015; Creel et al., 2013; Goymann and Wingfield, 2004; Sapolsky, 2005; Sapolsky and Ray, 1989). Dominating a social interaction may elicit a weaker stress response and lead to a faster recovery to pre-conflict concentrations than being defeated (Koolhaas et al., 2011; Øverli et al., 1999). Being able to redirect aggression towards a lower-ranking third party after being defeated and having strong social bonds or coalitionary support may further serve as stress coping outlets and social buffer that enable individuals to downregulate physiological stress (Abbott et al., 2003; Creel et al., 2013; Sapolsky, 2005; Young et al., 2014). Low-ranking males, with relatively low dominance potential, may thus have less of a buffer to cope with socially-induced stress (DuRant et al., 2016; Romero et al., 2009), i.e., experience stronger physiological constraints. They should therefore be under selection pressure to adjust both their social and sexual behaviour in ways that minimise their exposure to conflicts and allow them to downregulate their physiological stress (Briffa and Sneddon, 2007; Goymann and Wingfield, 2004; Raulo and Dantzer, 2018; Ricklefs and Wikelski, 2002; Romero et al., 2009; Sapolsky, 2005; Stier et al., 2012; Teunissen et al., 2018). Whether and how rank-related asymmetries in dominance potential and physiological stress mediate rank-related variations in male reproductive investment, performance, and ultimately, reproductive success is currently unknown

(Beehner and Bergman, 2017; Cavigelli and Caruso, 2015; Dantzer et al., 2016; Moore and Hopkins, 2009; Ricklefs and Wikelski, 2002).

Most socio-endocrine studies that investigated the physiological costs of reproductive investment and mate competition were conducted on species where males adopt alternative reproductive tactics as a function of their social rank. For example, in many species, top-ranking males engage in mate-guarding – a tactic by which a male consorts with a receptive female and aggressively disrupts attempts by lower-ranking males to access or mate with the female – whereas lower-ranking males adopt tactics that avoid direct mate competition (Bergman et al., 2005; Corlatti et al., 2012; Gesquiere et al., 2011; Girard-Buttoz et al., 2014; Setchell et al., 2010). These studies emphasised the physiological costs borne by high-ranking, actively competing males and how these costs may constrain their performance and reproductive share within the social group (Emery Thompson and Georgiev, 2014; Port and Kappeler, 2010). Yet these studies provided limited insight into why subordinates usually invest less than dominant males in reproduction and adopt alternative reproductive tactics. In addition, the possibility that male reproductive investment may not only depend on the physiological costs of sexual activities and mate competition but also be constrained by the time allocated to – and the physiological costs of – other fitness-enhancing activities, such as social activities, has rarely been considered (Raulo and Dantzer, 2018; Rimbach et al., 2016).

Here, we investigated the influence of male dominance potential on the interplay between physiological stress and male investment in social and sexual activities in the spotted hyena (*Crocuta crocuta*). Spotted hyenas live in large clans characterised by a stable linear hierarchy (East and Hofer, 2001), fission-fusion system (Smith et al., 2008), and promiscuous breeding with no distinct breeding season (East et al., 2003; Engh et al., 2002). Males do not adopt alternative reproductive tactics depending on their social rank but do differ in the extent to which they maintain associations with females (East and Hofer, 2001; Szykman et al., 2001). Females have control over copulation (East et al., 1993; Szykman et al., 2007) and a male’s chance to be chosen as a sire primarily depends on whether he was a member of the clan when the female was born (Davidian et al., 2016; Höner et al., 2007), and on his investment in associating and fostering a relationship with the female (East et al., 2003; Szykman et al., 2001). Male reproductive success is strongly and positively correlated with male social rank (Davidian et al., 2016). Social rank and access to females is not determined by physical aggression (Curren et al., 2015; East et al., 2003; East and Hofer, 2001) but by asymmetries in the number of coalitionary supporters within a clan (Vullioud et al., 2019). Dominance relationships are reinforced through ritualised greetings (East et al., 1993; Smith et al., 2010a), low-intensity agonistic behaviours, and dominance displays between pairs of individuals or coalitions (Curren et al., 2015; Smith et al., 2010b). Dispersing males acquire the lowest social rank in the new clan upon immigration and increase in social rank with increasing tenure, when a male occupying a rank above them dies or (re)disperses (East and Hofer, 2001). These males remain subordinate to native females and reproductively active native (hereafter 'philopatric males') males (Holekamp and Smale, 1998; Vullioud et al., 2019). Because of the asymmetries in social bonds and coalition partners between males, and the rigidity of the linear social hierarchy, high-ranking males have more opportunities to dominate other males and have more stress coping outlets than low-ranking males; high-ranking males therefore have a higher dominance potential than low-ranking males (Curren et al., 2015; East and Hofer, 2001; Goymann and Wingfield, 2004; Smith et al., 2010b; Vullioud et al., 2019).

We approximated a male's dominance potential by his standardised social rank, i.e., his position in the hierarchy relative to that of the other reproductively active males in the clan. The standardised rank is an integrated measure of the challenges a male receives and the coping opportunities he has (see methods for more details). We combined measurements of faecal glucocorticoid metabolite concentrations (fGMC) as a proxy of physiological stress with behavioural data from males living in eight social groups ('clans') of a free-ranging population. We tested whether (1) social and sexual interactions influence male physiological stress, (2) male dominance potential influences physiological stress in these contexts, and (3) rank-related differences in physiological stress in turn affect male sociality, reproductive investment and how males allocate their reproductive effort among females of different reproductive value.

We predicted that physiological stress should decrease with increasing male standardised social rank and associated dominance potential when males were socially active and when they sexually invested in females and concurrently interacted with male competitors. We further predicted that physiological stress should not vary with male standardised social rank when males did not interact with other males (e.g., when alone and when sexually investing in females in the absence of male competitors), and that physiological stress should be lowest when males spent time alone. If so, low-ranking males should experience stronger physiological constraints than high-ranking males and adjust their behaviour accordingly: they should spend relatively more time alone, conversely invest less in social and sexual activities, and focus their reproductive investment on females of low reproductive value (Hofer and East, 2003), for which there is less competition for access (East and Hofer, 2001; East et al., 2003; Szykman et al., 2007). Finally, we predicted that philopatric males should benefit from their relatively high dominance potential (Davidian et al., 2016) i.e., weaker physiological constraints and native origin, i.e., pre-established social bonds with relatives and high level social integration (Vullioud et al., 2019) in terms of higher investment in sexual activities than immigrant males.

Methods

Study area and population

All hyenas of the eight resident clans inhabiting the 250-km^2 floor of the Ngorongoro Crater (3°11'S, 35°34'E) in Tanzania were monitored between April 1996 and November 2015. The hyenas were individually recognised by their unique spot pattern and ear notches. Their age was estimated based on pelage characteristics, body size, locomotory abilities, and behavioral development (Höner et al., 2010). Dispersal is strongly male-biased; approximately 85% of males leave their natal clan and immigrate into another Crater clan to breed (Davidian et al., 2016; Höner et al., 2007), whereas virtually all females remain in their natal clan throughout their life (Höner et al., 2005). Male breeding group choice and dispersal decision are driven by the distribution of breeding partners (i.e., young females) across natal and nonnatal clans, rather than the number of competitors, the number of unrelated females, or the prey abundance in the clan territory (Davidian et al., 2016; Höner et al., 2007). Because extra-clan paternity is extremely rare (Davidian et al., 2016), male breeding prospects are influenced by their clan membership and the number of young females in the clan (Höner et al., 2010, 2007). The number of adult (≥ 24 months old) clan members and reproductively active males increased during the study period from 15.5 ± 3.4 to 42.9 ± 5.9 and from 4.0 ± 1.1 to 13.1 ± 2.2, respectively.

Male dispersal status

We defined philopatry and dispersal as the outcome of breeding-clan selection that led a male to choose and start his reproductive career in his natal clan or in another clan, respectively (Davidian et al., 2016). We considered a male to have chosen a clan when he expressed sexual behaviour toward females and/or invested in joining the social hierarchy of sexually active males in the natal clan (‘philopatric’ male) or another clan (‘immigrant’ male) for at least 3 months (Davidian et al., 2016). The date of clan choice was the date of first observation of such behaviour for philopatric males and of first sighting in the new clan territory for dispersers, and defined the start of a male’s tenure. Males who did not show any sign of sexual activity or had not met the 3-month criterion before their date of last sighting or the end of the study period were excluded from the analyses. By applying these criteria instead of an age-based categorisation of males, we avoided potential biases in male behaviour and physiology between philopatric and immigrant males that could arise from differences in sexual motivation and maturity (Akinyi et al., 2017; Bercovitch, 1986; Davidian et al., 2016; Höner et al., 2008). During the study period, 264 males dispersed to another Crater clan, 42 Crater-born males started their reproductive career in their natal clan, and 28 males immigrated into Crater clans from elsewhere. We considered both first-time selectors and males who engaged in breeding dispersal, that is, males who (re)dispersed from their clan of first choice and immigrated into another Crater clan (secondary clan choice) (Davidian et al., 2016). As a consequence, philopatric males can become immigrants and the social rank of immigrant males does not strongly correlate with their age.

Social rank and dominance potential

Individual social rank was determined on the day of collection of faecal and behavioural data. We determined individual social rank in two steps. First, we constructed the social hierarchy of each clan by assigning ordinal ranks to clan members based on the outcome of dyadic agonistic interactions (Goymann et al., 2003b; Vullioud et al., 2019). The individual with the highest proportion of wins was assigned the ordinal rank 1 and the one with the lowest proportion of wins was assigned the ordinal rank equal to the number of clan members (e.g., ordinal rank = 20 if N = 20 clan members). Second, we scaled (‘standardised’) these ordinal ranks to the number of clan members, using the formula: Standardised rank_i = ([# of clan members – ordinal rank_i]/([# of clan members – 1]/2)) – 1. Male standardised rank was calculated considering as clan members all philopatric and immigrant male members of the focal clan. Female standardised rank – here, used as a proxy for female relative reproductive value (Davidian et al., 2016; Hofer and East, 2003) – was calculated considering as clan members all sub-adult (≥12 months old) and adult clan members of both sexes born in the focal clan. Standardised rank ranges from −1 (lowest rank) to +1 (highest rank). Individuals with standardised rank within the top, middle, and lowest third of the total range were classified as high-ranking, medium-ranking, and low-ranking, respectively. Using standardised ranks instead of ordinal ranks has several advantages regarding our study system and questions. In contrast to ordinal ranks which remain unchanged when new immigrants join the clan at the bottom of the hierarchy, standardised ranks account for all demographic changes within a clan and for differences in the number of clan members between clans. Standardised rank reflects the proportion of clan members ranked above and below a focal individual; for example, a medium-ranking male with a standardised rank of 0 will dominate ca. 50% and submit to ca. 50% of males of his clan. Standardised rank can thus be used as an integrated measure of the ratio of dominance and coping opportunities (i.e., display dominance, employ scapegoating, enjoy coalitionary support and social buffer) to the challenges received by an individual, that is, male dominance potential. Furthermore, using standardised rank circumvents the main conceptual pitfall for interpretation – namely the “direction of causality” – associated with the use of ordinal ranks, that is, that physiological and behavioural patterns obtained by correlational studies can be interpreted either as a cause or a consequence of a given ordinal rank and associated physiological, energetic, or nutritional states (Beehner and Bergman, 2017). Because standardised rank is a relational measure that depends on other group members, it is unlikely to be caused by an individual’s physiological and behavioural traits.

Faeces collection and hormone analysis

We measured fGMCs in 451 faecal samples from 147 males, including 60 samples from 15 philopatric and 391 samples from 132 immigrant males (median = 3 samples per male, range = 1 − 17). Faeces were collected immediately after defaecation, mixed, subsampled, and stored in liquid nitrogen until transported to Berlin (Germany) on dry ice where they were stored at −80°C until further processing. Faecal subsamples were freeze-dried prior to steroid extraction and fGMCs quantified using an ‘in-house’ competitive enzyme-linked immunosorbent assay (ELISA) based on an antibody generated against cortisol-3-CMO that was previously validated analytically and physiologically for spotted hyenas (Benhaiem et al., 2012). Aliquots were extracted and assayed in a single batch; measurements were performed in duplicates and results were accepted only when their coefficient of variation (CV) was ≤5%. Intra-assay and inter-assay CVs were 2% and 15% for the low faecal control pool, and 1% and 8% for the high control pool, respectively (for more details on extraction and assay protocol, see (Davidian et al., 2015). Measurements are expressed as nanogram per gram (ng/g) of dry faecal matter. Concentrations of hormone metabolites in faeces provide an integrated measure of the endocrine activity over a period of hours to days (Goymann, 2005), that is, they reflect the combination of baseline concentrations, endocrine response to social conflict (of potentially varying amplitude and degree of repetition), post-conflict recovery (of varying latency), and downregulation by use of coping outlets and social buffer (see also "allostatic load"; Goymann and Wingfield, 2004). For analyses of factors influencing physiological stress, we matched each faecal sample and associated fGMC to the behavior the male was observed to express during a minimum of 2 hours between 36 hours and 10 hours prior to defaecation. This period was defined based on the period during which the physiological response to a challenge induced experimentally is detected in faeces of spotted hyenas (Benhaiem et al., 2012). If a male had been monitored for ≤2 hours during that period, his activity was considered unknown, and the faecal sample was excluded from the model (N = 85 faeces with unknown male activity).

Behavioural and proximity data

We conducted behavioural observations throughout the day (between 0600 and 1900 h) at resting places, communal and birth dens, and other areas of clan territories. We collected behavioural and proximity (presence and number of other clan members) data for 334 males using all-occurrence and focal animal sampling, as well as hourly scans. Living in fluid fission-fusion societies, males and females spend time alone or in subgroups of varying size and composition (Aureli et al., 2008; Smith et al., 2008).

We categorised male activity as *'alone'*, *'social'*, or *'sexual'* using definitions adjusted to the two main analyses; both sets of definitions were conservative with respect to our predictions. In the analysis of the relationship between activity and physiological stress, males were considered alone when they were resting, travelling, hunting or feeding more than 50m away from other males – a distance at which males are unlikely to be the target of agonistic behaviours (East and Hofer, 2001) – or when they were within 50m from male clan members but not engaging in any social or sexual activity. Males were considered socially active when they were involved in affiliative and/or agonistic interactions with other males such as greeting, social sniffing, scapegoating, coalition (as emitter or target), and dominance displays in the context of feeding (East et al., 1993; Smith et al., 2010b), and when they did not engage in any sexual activity. Scapegoating refers to a frequent, conspicuous behaviour whereby the target of a dominant act immediately redirects dominance (e.g., approaches with its tail up) onto a bystander of lower social rank. Scapegoating often takes the form of a 'cascade' where successive targets redirect dominance onto a lower-ranking third party. When no lower-ranking individual is present, the lowest-ranking individual involved in such cascades may occasionally redirect dominance towards a smaller carnivore, bird, rock or even the research vehicle. Sexual activities comprised repeated intersexual investigatory behaviours, repeated short bouts of approach-retreat courtship displays, prolonged following of a female ('shadowing'), harassment, and mating (Holekamp and Smale, 1998; Szykman et al., 2007). We considered two contexts of sexual activity; (i) '*sexually active with others*', whenever the focal male exhibited sexual behaviours towards a female and concurrently interacted with other males in close proximity – typically within the context of intrasexual competition over access or most proximate position to a female (East and Hofer, 2001; Goymann et al., 2003a) – and (ii) '*sexually active alone*', when the focal male engaged in sexual activities but did not concurrently interact with other males or when no other male was present within 50m. To assess whether different types of sexual behaviour are associated with different physiological stress – e.g., due to differential level of male sexual arousal (Koolhaas et al., 2011) – we compared the fGMCs of males who displayed the two main types of sexual behaviour, namely, approach-retreat courtship displays and shadowing. We found that both types of sexual behaviour were associated with similar fGMCs when males were sexually active alone – a context that allows to discard potentially confounding effects of intrasexual competition – and when fGMCs were matched by male identity and social rank (Wilcoxon signed-rank test; $V = 26$, $P = 0.73$, $N = 9$; median of between-group differences = 0.76 ng/g, $CI_{95\%} = -7.89 - 22.19$ ng/g). We therefore did not distinguish between the types of male sexual behaviour in our analyses.

For the analysis investigating the determinants of male activity budget and their exposure to mate competition, we categorised male activity based on their proximity to other clan members (males and females); a focal male was considered '*alone'* when no other clan member was present within 50m, '*socially active*' when at least one other clan member was present within 50m but the focal male did not engage in sexual activities, '*sexually active alone*' when the male engaged in sexual activities but no other male was present within 50m, and '*sexually active with others*' when the male engaged in sexual activities and at least one other male was present within 50m. These data were converted into hourly scans ('sightings') of activity and proximity data (median = 47 sightings per male, range = 6 – 349) for the analyses. Previous studies showed that male spotted hyenas most frequently associate and socially interact with other males and that social interactions between males and females are comparatively rare (East et al., 1993; Ilany et al., 2015). Males are also as likely to associate with males that occupy a rank immediately above them as they are to associate with a male ranked immediately below (Smith et al., 2007). Thus, when males interact with other males, the type and direction of behaviours they engage in should generally match those predicted by their dominance potential.

Spotted hyenas most often hunt alone and feed in groups (Holekamp et al., 1997; Smith et al., 2008) at night (Kruuk, 1972). During daytime, hunting and feeding make up less than 5% of all sightings (Smith et al., 2008) and males of all social ranks hunt at similar rates (Holekamp et al., 1997). Our behavioural data, in particular the proportions of time spent alone and with other clan members, are therefore unlikely to be driven by (i) hunting and feeding patterns or (ii) differential patterns of hunting and feeding across social ranks. Furthermore, differences in physiological stress between low-ranking and high-ranking male hyenas are unlikely to arise from rank-related nutritional status. Previous studies found that food deprivation can either induce prolonged, elevated physiological stress or an increased amplitude of the stress response in socially challenging contexts, and thereby reveal or amplify differences between high-ranking and low-ranking individuals when nutritional status is correlated with social rank (Killen et al., 2013). In spotted hyenas, however, there is currently no empirical evidence that low-ranking males suffer from food deprivation; low-ranking and high-ranking males have similar body size and body mass (East and Hofer, 2001; Engh et al., 2002), and engage in similar hunting rates (Holekamp et al., 1997).

Statistical analyses

Statistical analyses were conducted using R software v.3.4.3 and associated packages (R Core Team, 2017). Data are presented as means ± SD unless stated otherwise. The threshold for significance (P-value) was set to 5% for deterministic statistical analyses.

Influence of male dominance potential and activity on physiological stress

We assessed the factors that influence fGMC using a general linear mixed-effects model (GLMM; function *lmer()* in package *lme4*; Bates et al., 2015). fGMCs were normalised using natural-logarithmic transformation prior to analyses. Covariates included male standardised rank, the type of activity (four categories: 'alone', 'social', 'sexual (alone)' and 'sexual (with others)'), the interaction between male standardised rank and activity, and male origin, that is, whether the male had grown up in the focal clan ('native', for philopatric males) or another clan ('foreign', for immigrant males). We additionally controlled for male age and the amount of faeces in a sample (four categories; 'small' [≤150 g], 'medium' [150 g – 300 g], 'large' [>300 g] and 'unknown'). Faeces amount was considered as a covariate to control for potential "dilution effects" on fGMC (Goymann, 2012). We included male identity as an individual-level random factor. Regression coefficients were estimated by maximum likelihood using Laplace approximation. Significance of effects was assessed as the marginal contribution of each covariate to the full model by subtracting the likelihood of the reduced model without the specific covariate from the full model; P-values were calculated using parametric bootstrapped likelihood ratio tests with 1000 simulations (package *pbkrtest*) (Halekoh and Højsgaard, 2014). Model residuals satisfied the assumptions of normal distribution and homogeneity of variances (residual plots), and did not show signs of multi-collinearity between fixed effects (correlation matrix and squared generalised variance inflation ratios) (Fox and Monette, 1992). We further assessed the significance of the relationship between male standardised social rank and fGMC for each type of activity (slope comparison to zero) and compared the slopes (contrast analysis) when males were alone *vs.* socially active and when sexually active (alone) *vs.* sexually active (with others), using the function *emtrend()* in package *emmeans* (Lenth, 2018); P-values for the contrast analysis were adjusted for multiple comparisons using Bonferroni corrections.

Influence of rank-related physiological stress on activity budget

We investigated the factors influencing male activity budget using a Bayesian multinomial logistic mixed-effects model based on Hamiltonian Monte Carlo algorithms. We used the packages *rstan* (Stan Development Team, 2017) and *rethinking* (McElreath, 2016) following the procedure as previously implemented (see Koster and McElreath, 2017) for detailed procedure and R script). This model recognises time as a limited resource and that the time a male allocates to a given activity inherently reduces the time it can allocate to other activities, enabling the investigation of time-based behavioural trade-offs (Koster and McElreath, 2017). Here, we considered that at each hourly sighting (N = 21970), a focal male could express one of three categorical behavioural responses: be alone, be sexually active, or be socially active. Fixed effects were male standardised social rank (as a proxy for dominance potential and associated physiological constraints), age, and origin; male identity (N = 334) was included as a random factor. The multinomial model contrasted the odds of expressing a given behaviour relative to the reference behaviour (here, 'alone'). The coefficients estimated by the model were log(odds) and were converted into odd ratios, using the formula: exp[coefficient]. Odd ratios >1 and odd ratios <1 indicate a relative increase and decrease, respectively, in the likelihood to express the behaviour. We considered a fixed effect significant if the credible interval ($\mathrm{CrI}_{95\%}$) of its estimated coefficient did not include 1. The model also has the advantage of providing information on the variance of individual-level random effects for each behavioural response as well as their correlation across responses, thereby potentially revealing if individuals who regularly engage in one activity also invests relatively more or less in another activity (Koster and McElreath, 2017). Because the multinomial model examines the proportional allocation of time to social and sexual activities relative to the reference behaviour, the total time considered for each fit corresponds to a subset of all possible behavioural responses and of the activity budget; model estimates thus do not provide information on the effect of the covariates on the overall proportion of total time males allocated to each activity (i.e., relative to the combined time allocated to all other activities). We therefore investigated the extent to which male standardised rank and origin influenced male probability to allocate time to each activity, using the model predicted probabilities and their 95% percentile intervals, as calculated from the posterior samples of the Bayesian, multinomial logistic mixed-effect model (Koster and McElreath, 2017).

Male exposure to intrasexual competition and quality of male sexual investment

We investigated the factors that influenced male exposure to intrasexual competition when sexually active using hourly counts of the number of males in proximity to a focal male and his target female. Because the data were both overdispersed and zero-inflated, we conducted a hurdle model using the function *glmmadmb()* in package *glmmADMB* (Fournier et al., 2012). Such a procedure allows to analyse overdispersed, zero-inflated count data in two steps, each step specifying a distinct underlying process: (i) a binomial process to investigate the factors influencing the presence ('*sexual (with others)*' coded as '1') *vs.* absence ('*sexual (alone)*' coded as '0') of males in proximity to a focal male (2034 sightings of 218 males) and (ii) a truncated count process – here, a truncated negative binomial model to circumvent the overdispersion of residuals – restricted to counts with value ≥1, to assess the factors that influenced the number of proximate males when focal males were sighted in the context '*sexual (with others)*' (1399 sightings of 197 males). Covariates included male standardised rank and origin as well as the standardised rank of the courted female; male identity was considered as a random factor. Finally, we performed a logistic GLMM to assess the effects of male standardised social rank and origin on male likelihood to sexually invest in females of high standardised social rank, that is, of high reproductive value, as opposed to females of low and medium rank.

Results

Relationship between male fGMC, social rank and activity

There was a significant effect of the interaction between male standardised rank and the type of activity males engaged in on their fGMC (LR = 14.70, P = 0.006; whole model: LR = 128.74, P < 0.001, N = 366 faeces with matched activity from 121 males; see Table S1 for detailed model estimates). fGMC decreased with increasing standardised rank when males were socially active ('social'; slope coefficient = −0.51, $CI_{95\%}$ = −0.78 to −0.24, P < 0.001; contrast slope alone – social = 0.39, t-ratio = 2.41, P = 0.033; Figure 1; Table 1) and when males were sexually active and concurrently interacting with other males ('sexual with others'; slope coefficient: −0.29, $CI_{95\%}$ = −0.55 to −0.02, P = 0.037; contrast slope $\text{sexual}_{\text{alone}}$ - $\text{sexual}_{\text{with others}}$ = 0.53, t-ratio = 2.54, P = 0.023). In contrast, male standardised rank did not influence fGMC when males did not interact with clan members ('alone'; slope coefficient =

−0.12, $CI_{95\%}$ = −0.37 − 0.14, P = 0.37) and when males were sexually active without interacting with other males (‘sexual alone’; slope coefficient = 0.25, $CI_{95\%}$ = −0.12 − 0.61, P = 0.19). Male origin had a significant effect on fGMC, with native males having higher fGMC than foreigners (LR = 4.55, P = 0.046; Table 1).

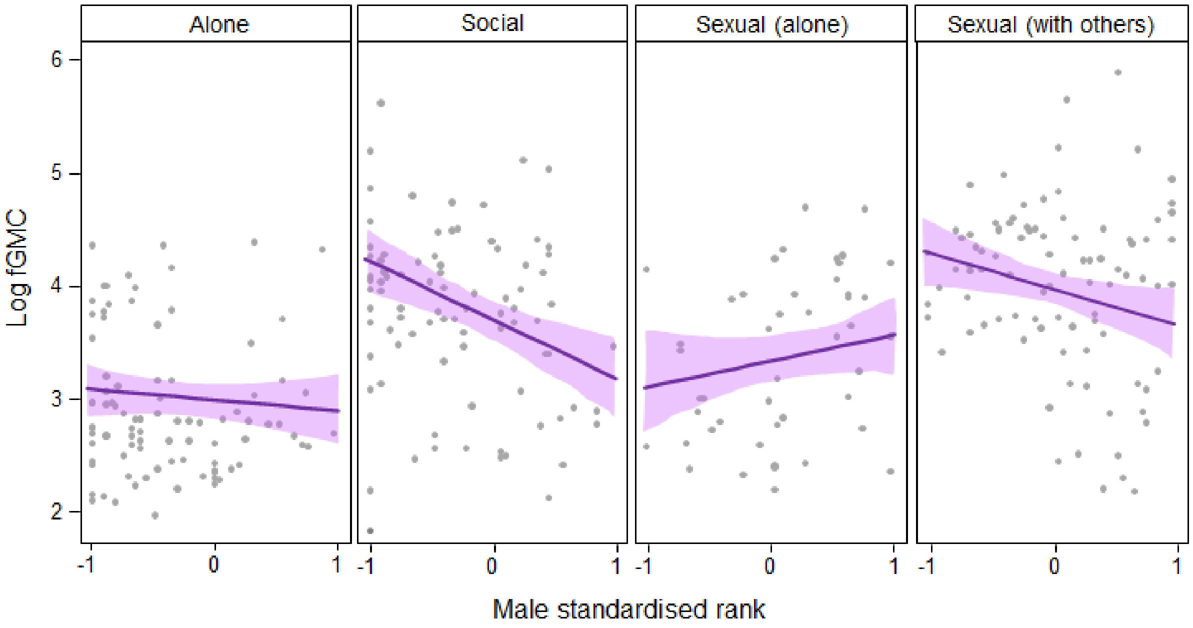

Figure 1 | The influence of male activity and standardised rank on physiological stress. Purple lines depict the relationship between social rank and faecal glucocorticoid metabolite concentrations (fGMC) in natural log units. Shaded areas indicate 95% confidence intervals as predicted by a general linear mixed-effects model when other covariates were held constant (age: population mean = 6.69 years, origin: foreigner, faeces amount: medium) and without accounting for individual-level random effects. Grey dots correspond to raw data (in log [ng/g]). Standardised rank is used as a proxy for male dominance potential and ranges from −1 (lowest rank) to +1 (highest rank).

Table 1. Predicted and observed (raw) physiological stress as a function of male social rank and origin. Values correspond to mean faecal glucocorticoid metabolite concentrations in ng/g. Predicted means ($CI_{95\%}$) are back-transformed predictions for the combined influence of male social rank and origin as derived from a general linear mixed-effects model when other covariates were held constant (age: population mean = 6.69 years, faeces amount: medium) and without accounting for individual-level random effects. Low-ranking and high-ranking immigrants are foreigners with standardised rank between −1 and −0.33 and between 0.33 and 1, respectively. Philopatric males are native males with high social rank. Predicted means were computed using the mean standardised social rank of each male category (low-ranking immigrant: mean = −0.74; high-ranking immigrant: mean = 0.64; philopatric male: mean = 0.79).

	Low-ranking immigrant		High-ranking immigrant		Philopatric male	
Male activity	Predicted	Raw	Predicted	Raw	Predicted	Raw
Alone	20.9 (17.2 - 25.3)	25.2 ± 17.9	17.9 (14.2 - 21.9)	31.4 ± 27.7	25.2 (19.2 - 33.8)	33.2 ± 25.2
Social	54.1 (44.3 - 65.9)	61.8 ± 52.3	27.8 (21.2 - 34.8)	37.0 ± 28.5	35.1 (27.7 - 46.5)	48.9 ± 32.1
Sexual (alone)	22.6 (15.7 - 34.4)	23.8 ± 16.9	32.0 (22.7 - 41.5)	46.9 ± 20.3	45.8 (35.6 - 60.8)	83.4 ± 54.8
Sexual (with others)	62.6 (48.1 - 78.2)	69.9 ± 26.5	43.1 (31.8 - 54.5)	59.6 ± 43.3	54.6 (41.1 - 74.3)	104.4 ± 124.3

Rank-related investment in social and sexual activities

As male standardised rank increased, the odds improved that males allocated time to social activities (Bayesian multinomial mixed-effects logistic model; odd ratio = 1.49; $CrI_{95\%}$ = 1.40 – 1.57) and to sexual activities (odds ratio = 1.86; $CrI_{95\%}$ = 1.65 – 2.06) relative to being alone, and that the relative increase in sexual investment was higher than the increase in social activities. Male origin also influenced male behaviour: native males were less likely to engage in social activities (odds ratio = 0.60; $CrI_{95\%}$ = 0.48 – 0.76) but were more likely to be sexually active (odds ratio = 1.74; $CrI_{95\%}$ = 1.17 – 2.57) than to be alone, as compared to foreigners (Table S2). Investigation of individual-level random effects further revealed (i) a relatively low variance for social activities (exp(estimate) = 1.40) in comparison to sexual activities (exp(estimate) = 2.29), and (ii) a significant positive correlation between male investment in social and sexual activities (0.30; $CrI_{95\%}$ = 0.11 – 0.50), when controlling for the fixed effects. The heterogeneity of random variance indicates that all males had similar propensities to allocate time to social activities but differed greatly in their propensity to allocate time to sexual activities. The positive correlation between social and sexual investment indicates that males with a relatively high propensity to invest in social activities also had a high propensity to invest in sexual activities. Inspection of the probabilities predicted by the model revealed that male social rank and origin both are strong determinants of male activity budget (Figure 2 and Figure 3a). As males increased in social rank, they spent less time alone and more time with social and sexual activities. Native males spent more time alone, less time in proximity to clan members, but invested more time in sexual activities than foreigners (Figure 2 and Figure 3a). Overall, males spent most of their time in proximity to other clan members ('social'; mean percentage out of total sightings: 73% ± 15%, N = 334 males), less time alone (20% ± 15%), and least of their time sexually active (7% ± 8%; Figure 3a).

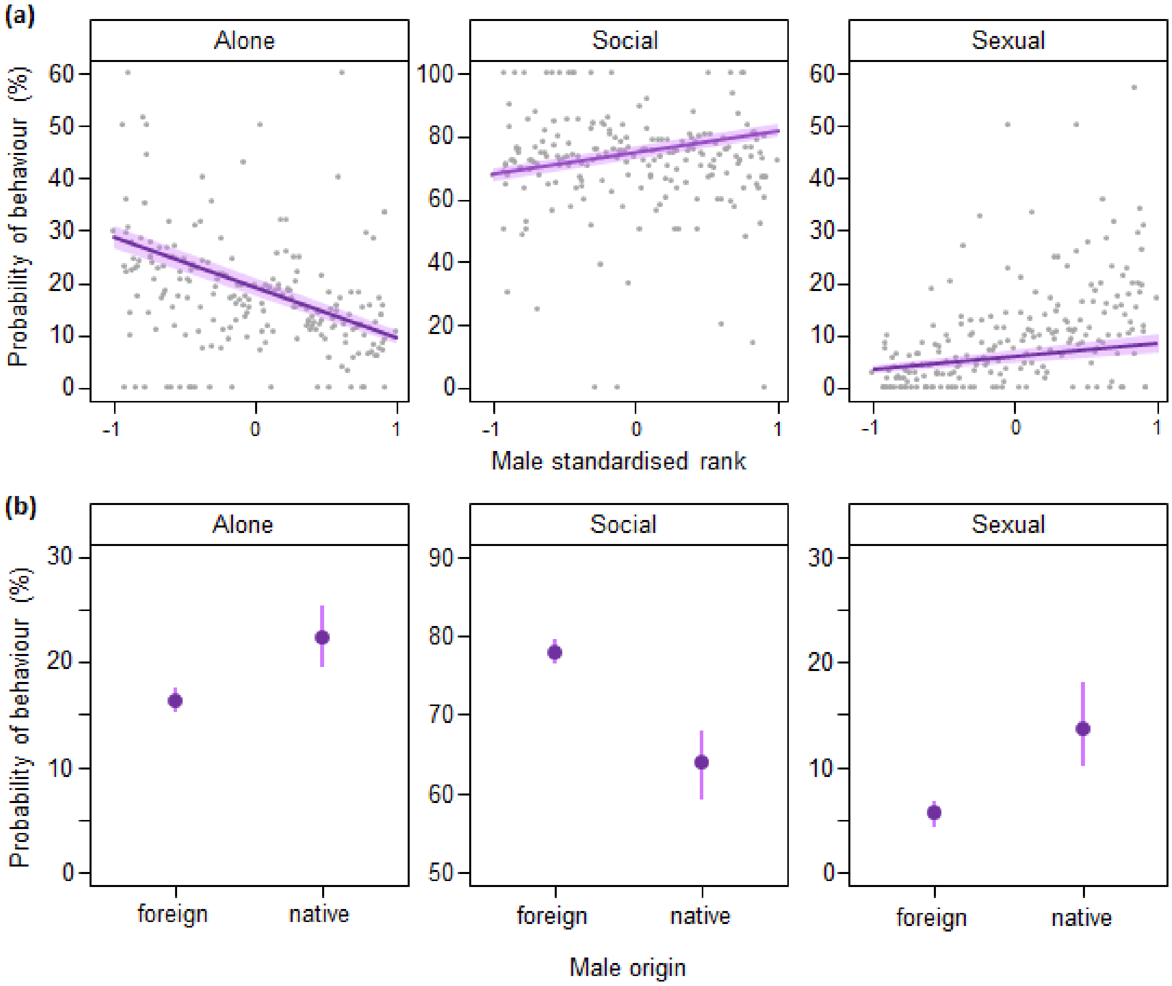

Figure 2 | The probability of males to engage in three activities as a function of their standardised rank (a) and origin (b). Probabilities are predicted means (purple lines and dots) with 95% percentile intervals (shaded areas and vertical bars) as calculated from the posterior samples of a Bayesian multinomial logistic mixed-effects model. Predictions were computed by holding covariates at their sample mean and without accounting for random effects (here, male identity). In **(a)**, predictions were computed for males of foreign origin. Grey dots correspond to observations, averaged at each 0.05 interval of standardised social rank for visibility. The scale on the y-axis was adjusted to the range of observations and differs between activities.

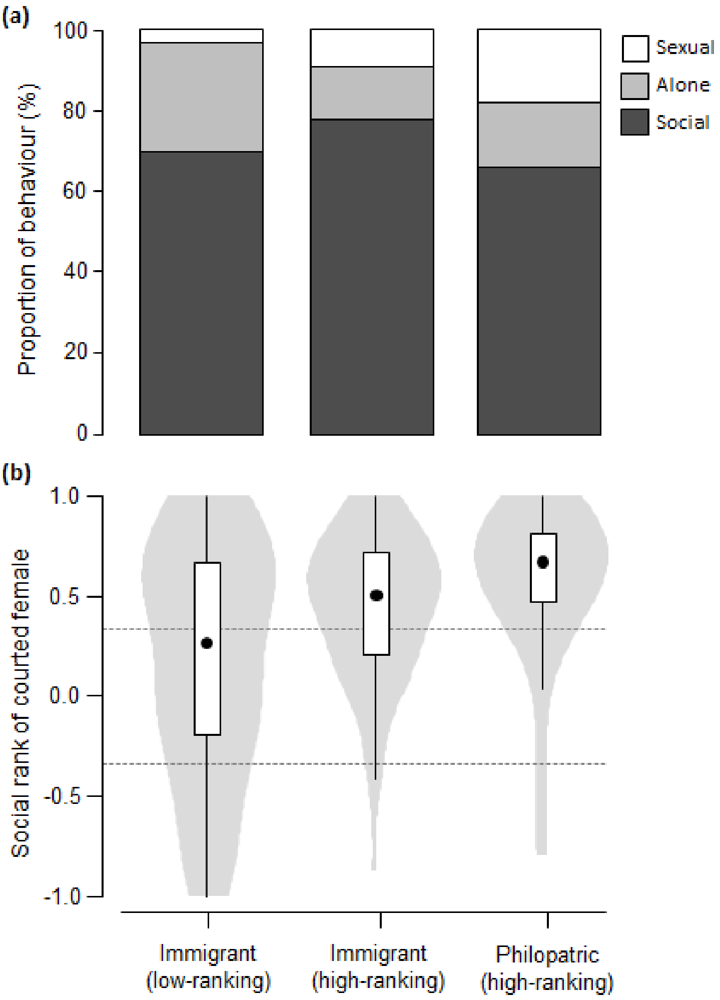

Figure 3 | The allocation of time to different activities and the social rank of the courted females as a function of male social rank and origin. (a) Observed mean proportion of time allocated to each activity. **(b)** Social rank of the females that were courted by males. Social rank was standardised and ranges from −1 (lowest rank) to +1 (highest rank). Dashed horizontal lines delimitate the range of standardised ranks within the top, middle, and lower thirds of the total range; these ranges comprise high-ranking, medium-ranking, and low-ranking females, respectively. Boxes indicate the interquartile range around the median (black dot), vertical bars represent female social ranks that lie within 1.5 times the interquartile range, and grey shaded areas represent the distribution (kernel density estimate) of the data.

Female quality and male exposure to intrasexual competition

Previous studies showed that high-ranking female hyenas were courted by males at higher rates than low-ranking females (East and Hofer, 2001; Szykman et al., 2007), suggesting that male-male competition for access to females may increase with increasing female social rank and associated reproductive value. We therefore tested whether female social rank and male characteristics influenced the presence and number of male competitors in close proximity to a male courting a female. When a male was sexually active, the likelihood that competitors were present (‘Presence model’) and the number of competitors (‘Count model’) that were present strongly increased with increasing social rank of the courted female (Table 2). Male social rank did not have a significant influence on either the presence or the number of competitors in close proximity (Table 2). Male origin had a significant effect on the presence of competitors, with native males being more likely than foreigners to be sexually active in the presence of competitors (Table 2).

When a male was sexually active, the likelihood that he courted a high-quality, high-ranking female was strongly influenced by male standardised rank (logistic GLMM: LR = 7.39, P = 0.007) and origin (LR = 8.97, P = 0.003; whole model: LR = 25.82, P < 0.0001, N = 2034 sightings from 218 males; see Table S3 for detailed coefficients). Low-ranking immigrants invested in females of lower quality (mean female standardised rank = 0.17 ± 0.59, N = 76; Figure 3b) than did high-ranking immigrants (0.44 ± 0.37, N = 78; Mann-Whitney U test; U = 2228.5, P = 0.008), and high-ranking immigrant males overall invested in lower-ranking females than did philopatric males (0.55 ± 0.41, N = 36; U =1055, P = 0.034).

Discussion

Our findings are consistent with the predictions of the hypothesis that rank-related physiological stress, arising from differences in social challenge and availability of coping outlets, is a key proximate mediator of variation in reproductive investment and success in male spotted hyenas.

Consistent with our predictions, physiological stress was similar for males of all social ranks when they were on their own or when they showed sexual interest in a female in the absence of male competitors. Furthermore, when males engaged in social interactions and when they competed with other males over access to a female, low-ranking males experienced higher physiological stress than when alone or sexually active alone, and their physiological stress was higher than that of high-ranking males. These results indicate that differences in physiological stress across male social ranks are contingent on interactions with other males and thus most likely arise from rank-related differences in male dominance potential.

Table 2 | Factors influencing the presence and number of competitors when males were sexually active. Shown are the regression coefficients and standard errors (s.e.) in log units, back-transformed coefficients as exp(coeff), and the corresponding likelihood ratio (LR) and P-values for each predictor variable. Regression coefficients were estimated by a hurdle model in two steps; a binomial mixed-effect model (whole Presence model: LR = 46.19, P < 0.001; individual-level random effect variance = 0.18; N = 2034 sightings from 218 males) and a zero-truncated negative binomial mixed-effect model (whole Count model: LR = 13.76, P = 0.003; individual-level random effect variance = 0.14; N = 1399 sightings from 197 males). For the Presence model, exp(coeff) correspond to odd ratios and indicate the factor of change in the likelihood to be sexually active with competitors relative to that of being sexually active without competitors present, with a one-unit increase in the predictor and when other covariates are held constant. For the Count model, exp(coeff) indicates the increase or decrease in the absolute number of competitors present with a one-unit increase in the predictor.

Fixed effect	coefficient	s.e.	z value	exp(coeff)	LR	P
Presence model						
Intercept	0.55	0.08	6.94	1.73		
Female social rank	0.54	0.09	5.95	1.72	35.10	< 0.001
Male social rank	-0.09	0.12	-0.80	0.91	0.64	0.425
Male origin (native)	0.56	0.20	2.88	1.75	8.20	0.004
Count model						
Intercept	1.06	0.04	24.49	2.89		
Female social rank	0.13	0.04	3.38	1.14	11.76	< 0.001
Male social rank	-0.09	0.05	-1.68	0.91	2.78	0.095
Male origin (native)	0.07	0.10	0.73	1.07	0.52	0.471

Previous studies predicted that rank-related physiological stress should mainly emerge in systems where dominance relationships are established and maintained through intense aggression and physical contest (Creel et al., 2013; Goymann and Wingfield, 2004). Our results demonstrate that intrasexual interactions can also elicit rank-related physiological stress in systems where dominance relationships are mostly determined by social conventions and where competition over social dominance and access to mates involves low levels of aggression.

Our findings further indicate that rank-related physiological stress can be substantial enough to induce costly disparities in sociality and reproductive investment between low-ranking and high-ranking males. Low-ranking spotted hyena males spent more time alone than did high-ranking males, at the expense of social and sexual activities. Furthermore, low-ranking males sexually invested in females of lower social rank – i.e., of lower reproductive value – than did high-ranking males. These tactics constitute an effective behavioural response by low-ranking males to avoid physiologically costly intrasexual competition and downregulate their physiological stress to levels similar to those of high-ranking males (as indicated by the lack of an overall correlation between physiological stress and male social rank; Figure S1, see also (Goymann et al., 2003b)). Reducing reproductive investment and focusing the investment on females of lower social rank however also reduce their opportunities to be chosen as mates and lowers their chance to mate with females of high reproductive value (East et al., 2003; Szykman et al., 2001). This may explain why low-ranking males have lower reproductive success than high-ranking males both in terms of the number and the reproductive value of the offspring they sire (Davidian et al., 2016).

Our results are consistent with the idea that male allocation of time to different activities is shaped by trade-offs between the benefits of these activities and the physiological and missed-opportunity costs associated with them (Briffa and Sneddon, 2007; Dunbar et al., 2009; Ricklefs and Wikelski, 2002). We found that all males spent most of their time in proximity to clan members, even low-ranking males for which social interactions elicited comparatively high physiological stress. This suggests that prominent investment in social interactions is compulsory for all males and may reflect the direct and indirect fitness benefits of these activities. In spotted hyenas, as in other group-living species, maintaining proximity to (and interacting with) clan members is essential for the social integration of males – that is, the establishment and maintenance of clan membership, social bonds, coalitions, and social rank – and can strongly influence male current and future reproductive success (Berghänel et al., 2011; East and Hofer, 2001; Gilby et al., 2013; Ryder et al., 2009; Smith et al., 2010a; Vullioud et al., 2019; Willisch et al., 2010; Wiszniewski et al., 2012; Young et al., 2013). Spending time with clan members may further help males locate females, provide males with opportunities to engage in sexual activities, and thereby increase their chances to be chosen as sires.

Two findings of our study strongly suggest that investment in reproduction by males is not only constrained by the physiological costs of sexual activities but also by the time and physiological resources they allocate to social activities. First, immigrant males spent more time in proximity to clan members but invested less in sexual activities than did philopatric males. These differences may stem from inherent differences in the level of social integration between philopatric and immigrant males. Philopatric males benefit from their native status in terms of stronger social bonds and greater social support than immigrant males (Vullioud et al., 2019). Philopatric males thus are better socially integrated in their clan than immigrants and may in turn afford to invest less in social activities in favour of an accrued investment in sexual activities (see also Günther et al., 2017; Ilany et al., 2015). The higher quantitative and qualitative reproductive investment by philopatric male spotted hyenas may explain why philopatric males start breeding at a younger age and sire a higher proportion of their offspring with females of high reproductive value than do immigrants (Davidian et al., 2016). Second, among immigrant males, an increase in social rank and dominance potential is associated with a decrease in physiological stress during social interactions and competition over access to mates. This reduces their need to spend time alone – to downregulate their physiological stress – and enables them to use this spare time and physiological resources to increase their investment in fitness-enhancing social and sexual activities (Dunbar et al., 2009; Pollard and Blumstein, 2008; Zera and Harshman, 2001). Furthermore, high-ranking males allocated most of their spare time and physiological resources to increasing their investment in sexual activities and focus on females of higher reproductive value.

Our findings highlight the importance of considering the time allocated to activities other than sexual activities and their associated physiological costs when studying the proximate causes of variation in reproductive investment and reproductive success across social ranks (see also Alonzo, 2010; Dunbar et al., 2009; Rimbach et al., 2016; van Noordwijk and van Schaik, 2004; Zera and Harshman, 2001). Patterns of rank-related physiological stress and reproductive investment are likely to vary depending on (i) factors that influence the nature and intensity of intrasexual competition – such as the seasonality of breeding and the availability of uncontested mates (Cant et al., 2006; Port and Cant, 2014), the nature of the contested resource, and whether males gain immediate or delayed fitness benefits (Cant et al., 2006; East and Hofer, 2001; Port and Cant, 2014; van Noordwijk and van Schaik, 2004) – and (ii) factors that influence the availability of stress coping outlets – such as the degree of cohesiveness of group members (Abbott et al., 2003; Aureli et al., 2008; Goymann and Wingfield, 2004; Sapolsky, 2005; Wroblewski et al., 2009). The ability of low-ranking male spotted hyenas to adjust their behaviour (i.e., stay alone, invest in uncontested, lower-quality mating partners) to their physiological constraints in ways that effectively balance their physiological stress mainly stems from the fluid fission-fusion dynamics of social groups in this species (Aureli et al., 2008; Smith et al., 2008). This and the fact that males compete over opportunities to foster relationships with females – a non-transferrable resource which provides delayed benefits (East et al., 1993; East and Hofer, 2001) **–** may explain the absence of rank-related alternative reproductive tactics among males in this species. In species where males engage in overt contest competition over rank and access to monopolisable oestrus females, social and sexual activities may incur high physiological costs (Cant et al., 2006; van Noordwijk and van Schaik, 2004). These costs may be particularly prohibitive for low-ranking males in spatially-cohesive species where opportunities to avoid or recover from social challenges and to access uncontested mates are scarce (Abbott et al., 2003; Goymann and Wingfield, 2004; Port and Cant, 2014; Sapolsky, 2005). This may drive the emergence of rank-related male alternative reproductive tactics in which low-ranking males either forgo reproduction, or adopt reproductive behaviours that circumvent or reduce the cost of intrasexual competition and reproductive investment within the group (e.g., opportunistic sneaky copulation, male-male coalition, or extra-group copulation; Isvaran and Clutton-Brock, 2007; Taborsky et al., 2008; Young et al., 2007).

Our study demonstrates that physiological stress plays a pivotal role in shaping male reproductive investment and reproductive skew. We show that male social rank, through dominance potential, mediates the physiological costs of social activities and competition over mates. This causes rank-related physiological constraints and behavioural trade-offs between the allocation of time and physiological resources to social integration, reproduction, and self-maintenance (Figure 4). With increasing dominance potential and level of social integration, males experience lower socially-induced physiological stress and increase their reproductive effort both quantitatively and qualitatively. They thereby create more opportunities to foster relationships with females, be in proximity to females when females reach estrus (i.e., higher ‘performance’; Figure 4), and increase their chances to be chosen as mates (i.e., higher reproductive success) (Langergraber et al., 2013; Szykman et al., 2001).

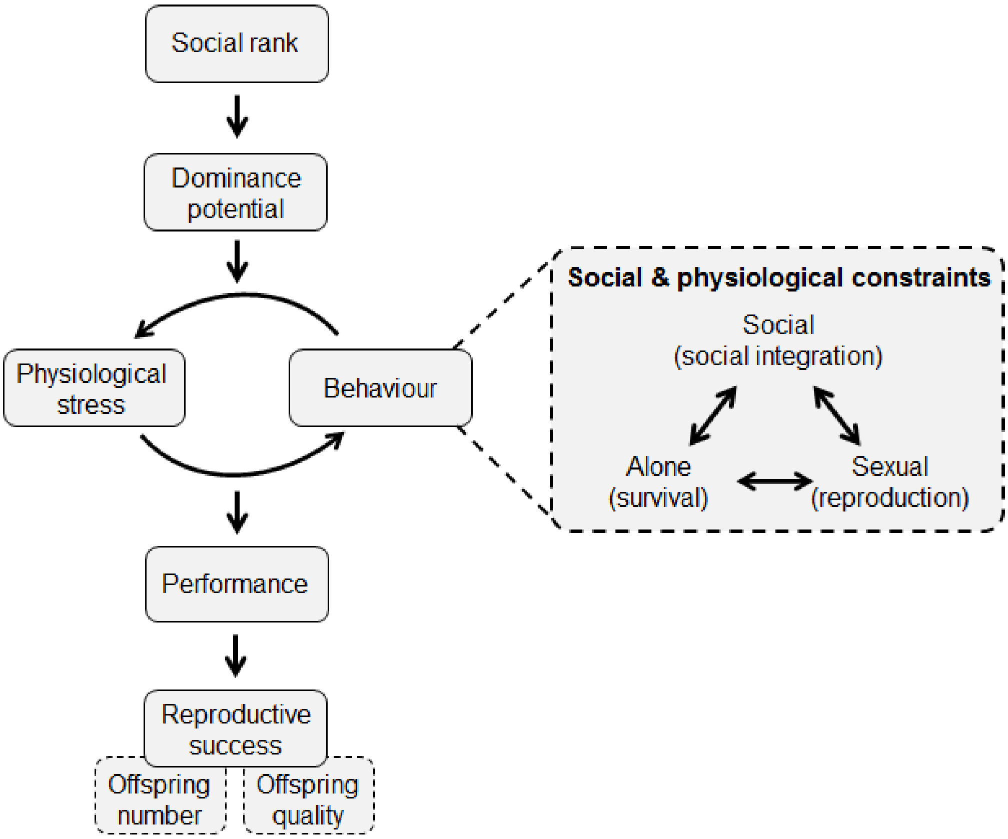

Figure 4 | The influence of social rank on the interplay between physiological stress and behaviour, and its consequences for male performance and reproductive success in the spotted hyena.

Our findings further provide insights into the social and physiological costs of dispersal – arising from loss of familiarity, social bonds and social rank upon immigration – and how these costs may contribute to the reduced reproductive success among recent immigrants, as reported in social and colonial mammals (spotted hyena: Davidian et al., 2016; feral horse: Debeffe et al., 2015; proboscis bat *Rhynchonycteris naso*: Günther et al., 2017; bighorn sheep *Ovis canadensis*: Poirier and Festa-Bianchet, 2018) and birds (barnacle goose *Branta leucopsis*: van der Jeugd, 2001; collared flycatcher *Ficedula albicollis*: Pärt, 1994). They may also explain why males of numerous mammals disperse in groups of socially bonded males or join groups containing familiar males, as a strategy to reduce these costs (Cozzi et al., 2018; Leimberger and Lewis, 2017; Schoof et al., 2009).

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Supplementary materials

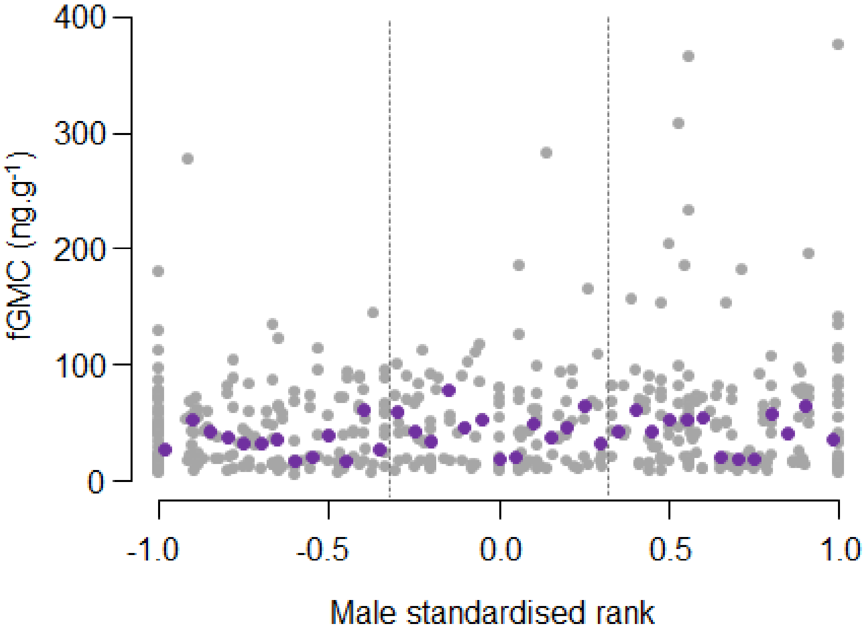

Figure S1| Relationship between male social rank and faecal cortisol metabolite concentrations in spotted hyaenas. Grey circles correspond to raw data for faecal cortisol metabolite concentration (fGMC) and corresponding male standardised social rank at defaecation (N = 451 faeces from 147 males). Purple circles correspond to the median fGMCs calculated at 0.05 standardised rank intervals (N = 39) which were used to perform a Pearson product-moment correlation (R = 0.18, df = 37, P = 0.28). Male standardised social rank ranges from −1 (lowest rank) to +1 (highest rank). Dashed vertical lines delimitate the range of standardised ranks within the top, middle, and lower thirds of the total range which were used to categorise males as high-ranking, medium-ranking, and low-ranking, respectively.

Table S1| The factors influencing male physiological stress. Shown are the regression coefficients and standard errors (s.e.) in natural logarithmic units, the corresponding percentage of change, t values, and corresponding likelihood ratios (LR) and P-values for each predictor variable. Coefficients indicate the change in faecal glucocorticoid metabolite concentrations (fGMC) relative to the intercept and the reference level for the categorical variables (activity: alone, origin: foreigner, faeces amount: medium). Regression coefficients were estimated by linear mixed model (whole model: LR = 128.74, P < 0.001; individual-level random effect variance = 0.02, N = 366 faeces from 121 males). %Change corresponds to the percentage of increase or decrease in the absolute value of fGMC (i.e., in $ng \cdot g^{-1}$) with a one-unit change in the value of the covariate compared to the reference, as calculated by [exp(coefficient)−1]×100. A one-unit increase in male standardised social rank encompasses a shift from low to medium and from medium to high rank categories.

Fixed effect	coefficient	s.e.	t value	%change	LR	P
Intercept	2.67	0.15	17.28			
Social rank	-0.12	0.13	-0.93	-11.3	0.86	0.680
Activity (Social)	0.66	0.11	6.05	93.5	76.08	0.009
Activity (Sexual alone)	0.34	0.13	2.62	40.5		
Activity (Sexual with others)	0.96	0.11	8.89	161.2		
Social rank * Activity (Social)	-0.39	0.16	-2.46	-32.3	14.70	0.006
Social rank * Activity (Sexual alone)	0.36	0.20	1.78	43.3		
Social rank * Activity (Sexual with others)	-0.17	0.16	-1.05	-15.6		
Origin (native)	0.32	0.14	2.24	37.7	4.55	0.046
Age	0.04	0.02	2.57	4.1	6.47	0.012
Faeces amount (Small)	0.19	0.11	1.83	20.9	7.50	0.068
Faeces amount (Large)	-0.20	0.13	-1.52	-18.1		
Faeces amount (Unknown)	-0.06	0.09	-0.59	-5.8		

Table S2| The effect of male social rank, origin and age on male investment in social and sexual activities. Shown are posterior mean as predicted by a Bayesian multinomial logistic mixed-effect model based on 21970 hourly sightings for 334 males. Results are presented as odd ratios, that is, exp(estimates), and indicate the effect of one-unit increase in standardised social rank and in age, and the effect of being native as compared to a foreigner on the time allocated to social or sexual activities relative to the reference category (here, being 'alone'). Odd ratios < 1 reflect a relative decrease and odd ratios > 1 a relative increase in the time allocated to an activity. A one-unit increase in male standardised social rank encompasses a shift from low to medium rank categories and from medium to high rank categories. Results for which credible intervals $CrI_{95\%}$ do not encompass 1 and were deemed significant.

Parameter	Behavioural response	
	social	sexual
Intercept	7.89 (6.14 – 10.27)	0.20 (0.13 – 0.32)
Social rank	1.49 (1.40 – 1.57)	1.86 (1.65 – 2.06)
Origin (native)	0.60 (0.48 – 0.76)	1.74 (1.17 – 2.57)
Age	1.04 (0.99 – 1.10)	1.64 (1.51 – 1.80)

Table S3| Variation in male probability to court a high-ranking female as a function of standardised rank and origin. Shown are the regression coefficients and standard errors (s.e.) in log units, odd-ratios, and the corresponding likelihood ratio (LR) and P-values for each predictor variable. Regression coefficients were estimated by a logistic mixed-effects model (whole model: LR = 25.82, P < 0.0001; individual-level random effect variance = 1.35; N = 2034 sightings from 218 males). Odd ratios indicate the factor of change in the likelihood to be sexually active with competitors relative to that of being sexually active without competitors present, with a one-unit increase in the predictor and when other covariates are held constant.

Fixed effect	coefficient	s.e.	z value	odd ratio	LR	P
Intercept	0.56	0.12	4.78	1.75		
Male social rank	0.43	0.16	3.02	1.54	7.89	0.007
Male origin (native)	1.03	0.34	2.70	2.80	8.97	0.003

CHAPTER 6

General discussion

The aim of this thesis was to identify proximate and ultimate drivers of variation in fitness among males in a social mammal, the spotted hyena, with particular emphasis on breeding-group choice and social rank, two traits that strongly influence the life history and social environment of group-living animals. To achieve this, I combined two decades of data on the social and sexual behaviour, physiology, survival and reproductive success of male spotted hyenas with demographic data from a population of eight free-ranging hyena clans.

6.1 Why do male philopatry and dispersal often coexist within a population?

Dispersal is one of the most important yet least understood drivers of ecological and evolutionary processes (Bowler and Benton, 2005; Ronce, 2007). In most group-living mammals, including the spotted hyena, most males disperse from their natal group and attempt to breed in another group; the remaining males either never disperse or only do so after a period of philopatry (Clutton-Brock, 2016; Greenwood, 1980; Höner et al., 2007). The evolutionary, ecological, and behavioural processes that lead to inter-individual variation in breeding-group choice, influence dispersal propensity and shape dispersal patterns at the level of the population are poorly understood (Bonte et al., 2012; Clobert et al., 2009; Clutton-Brock and Lukas, 2012).

In Chapter 3, I investigated the proximate and ultimate drivers of the coexistence of philopatry and dispersal in male spotted hyenas. I tested the two main hypotheses proposed to explain inter-individual variation in breeding-group choice and dispersal propensity within a sex. The first hypothesis is derived from dispersal theory and emphasises the costs of dispersal movements. It posits that only individuals of high phenotypic quality can pursue the most beneficial strategy; the others are left to do a “best-of-a-bad” job (Bonte et al., 2012; Bowler and Benton, 2005). The second hypothesis is derived from habitat selection theory and emphasises settlement decisions. It suggests that dispersers and philopatric males pursue the same strategy and choose the breeding habitat or group with the highest fitness prospects; philopatry and dispersal are then outcomes of these decisions (Boulinier et al., 2008).

My findings were consistent with the hypothesis that the coexistence of philopatry and dispersal is the result of a single process of breeding-group selection. Philopatric males and dispersers (i) were of similar phenotypic quality, (ii) applied the same criterion to choose and settle in a breeding clan and (iii) had similar survival and reproductive success after clan choice. I found no evidence to suggest that philopatric males and dispersers are two distinct male phenotypes that apply different strategies. The findings therefore were inconsistent with the hypothesis that philopatry is a "best-of-a-bad" strategy pursued by phenotypically inferior males.

6.1.1 The role of the distribution of breeding partners

In Chapter 3, I showed that philopatric males and dispersers both preferably joined clans with the largest number of potential breeding partners. Despite similar settlement preferences, philopatric males chose clans that contained on average half as many potential breeding partners as the clans chosen by dispersers. This apparent discrepancy is a consequence of the strong bias in clan quality in favour of nonnatal clans; males who chose a clan of low quality were simply more likely to have chosen their natal clan and established themselves as philopatric members than to have chosen a nonnatal clan and established themselves as immigrants.

The results in Chapter 3 demonstrated that the distribution of potential breeding females across natal and nonnatal clans is the main driver of dispersal patterns and of the coexistence of philopatry and dispersal among male spotted hyenas. The rules that females apply to choose their sires play a central role in shaping the observed patterns of breeding-clan choice by males. In contrast to females of other group-living species (Clarke and Faulkes, 1999; Clutton-Brock, 2016; Keane, 1990), the mate preferences of female spotted hyenas do not involve inbreeding avoidance *via* kin discrimination or discrimination based on the origin of males (East et al., 2003; Höner et al., 2007, 2008). This has two main implications for males: (i) males can breed in their natal clan and (ii) natal clans can occasionally contain the highest number of potential breeding partners in the population and philopatry can thus provide the highest fitness prospects. Furthermore, because female mate-choice rules are more restrictive for prospective philopatric males than for prospective dispersers, natal clans are less likely than any nonnatal clan to contain the highest number of potential breeding females in the population. The observed high proportion of dispersers

(85% of males) in the Ngorongoro Crater population was consistent with the strong bias in clan quality in favour of nonnatal clans (see also Höner et al., 2007).

6.1.2 The role of male social rank

In Chapter 3, I found that male social rank was a strong determinant of male reproductive success in spotted hyenas; males of high social rank had a higher annual reproductive rate and sired more offspring with females of high reproductive value. Previous studies had suggested that male reproductive success in hyenas is influenced by social rank and tenure (Engh et al., 2002; Holekamp and Smale, 1998). These studies, however, could not disentangle the effects of social rank and tenure because they only considered dispersers, for whom social rank and tenure are strongly correlated (East and Hofer, 2001). By combining data from philopatric males and dispersers in Chapter 3, I disentangled the effects of social rank and tenure. My study therefore represents the first robust assessment of the effect of social rank on male reproductive success in the spotted hyena. It further confirms previous evidence from the primate literature that social rank can be an important determinant of male reproductive success – and high-ranking males can exert considerable control over mating opportunities – even in species in which male contest competition for access to females is low, females exercise strong mate choice, and male ability to coerce females into mating with them is limited (Barbary macaques *Macaca sylvanus*: Young et al., 2013; rhesus macaques *Macaca mulatta*: Dubuc et al., 2011; Japanese macaque *Macaca fuscata*: Soltis et al., 2001; Verreaux's sifaka *Propithecus verreauxi*: Mass et al., 2009).

I further found that philopatric males gained reproductive benefits from their high social rank compared to immigrants: philopatric males sired their first offspring nearly one year earlier than did immigrants and they sired most (83% for philopatric males *versus* 39% for dispersers) of their offspring with females of high reproductive value. These findings strongly contrast with most empirical studies conducted thus far on group-living mammals where young philopatric males mainly gained indirect reproductive benefits through cooperative care of related offspring or delayed, direct benefits after queuing for dominant breeder status (Clutton-Brock et al., 2001; Creel and Waser, 1994; Doolan and Macdonald, 1996; Robbins and Robbins, 2005; Schradin and Lindholm, 2011). My study thus constitutes the first empirical evidence that philopatric males can reproduce as successfully as dispersers in a group-living mammal characterised by a strong dispersal bias. It further demonstrated that the reproductive benefits of philopatry increased the propensity of males to choose their natal group as breeding group, with nearly twice as many philopatric males as expected if choosing the natal clan did not yield any additional benefits compared to dispersal. Interestingly, philopatric males were more likely than dispersers to undertake secondary clan choice and did so after a shorter tenure than did dispersers. These results indicate that philopatric males may adjust the length of their tenure to the lower number of potential mates in the natal clan.

6.1.3 Consequences of breeding-group choices on lifetime reproductive success

The study presented in Chapter 3 focused on the reproductive consequences of philopatry and dispersal and provided estimates of male reproductive success during their tenure in their clan of first choice. As shown in Chapter 3 (see also Höner et al., 2007), a non-negligible proportion of males, i.e., approximately 18% of dispersers and 50% of philopatric males in the Ngorongoro Crater, undertake secondary clan choice (also termed ‘breeding dispersal’) during their lifetime. The causes and fitness consequences of secondary clan choice by male spotted hyenas, as well as males of other mammals, including primates (Jack and Fedigan, 2004; Wikberg et al., 2018), remain largely unknown. In spotted hyenas, males who (re-)disperse join their new clan at the very bottom of the social hierarchy; their reproductive trajectory may thus be very similar to that of first-time dispersers. Further studies investigating the reproductive output of secondary clan selectors would provide valuable insights into potential trade-offs and differences in lifetime reproductive success between philopatric males and dispersers who remained all their life in their clan of first choice, and males who undertook secondary clan choice.

6.1.4 Implications for studies on the evolution of dispersal and dispersal patterns

The study in Chapter 3 illustrates the benefit and relevance of studying dispersal and philopatry within the broader framework of habitat selection theory – that is, to emphasise the characteristics of available breeding groups and settlement choices – in order to derive predictions on the causes and fitness consequences of breeding-group choices and dispersal patterns. The study demonstrates that the coexistence of philopatry and dispersal among males within a population can be driven by the distribution of group qualities across natal and nonnatal groups of the population and by the reproductive benefits associated with philopatry, rather than high costs of dispersal preventing phenotypically inferior males to disperse. This study constitutes the first empirical demonstration that inter-individual variation in breeding-group choice and strong dispersal biases can emerge solely from the distribution and density of breeding partners when all individuals apply similar mate-based settlement rules (see Gilroy and Lockwood, 2016, 2012 for recent theoretical work). The study thereby demonstrates that high dispersal biases within a population or species do not necessarily result from a strong selective advantage of dispersal over philopatry.

Similar processes may explain the coexistence of male philopatry and dispersal in other group-living species. This is particularly likely to be the case in multimale, multifemale societies such as those of many primates, where social groups are large, reproductive skew is moderate and where dispersal distances by males are short. Recent empirical evidence demonstrated that, in such species, the genetic structure of the population is likely to be weak (Lukas et al., 2005; Quirici et al., 2011; Ross, 2001). This suggests that direct inbreeding avoidance by males and avoidance of kin competition are unlikely to be important drivers of male breeding-group choice and dispersal patterns in these systems.

6.2 By which mechanism does male social rank influence reproductive performance and success?

Understanding the causes and consequences of unequal partitioning of reproduction among members of a group is of major interest to gain insights into the evolution of group-living and the variability of social and breeding systems within and between species (Clutton-Brock and Huchard, 2013; Kappeler et al., 2013; Port and Cant, 2014). Physiological stress is a likely mediator of rank-related performance and reproductive success among males (Breuner et al., 2008; Dantzer et al., 2016; Moore and Hopkins, 2009). While there is substantial empirical evidence to demonstrate that male social rank and physiological stress can influence male reproductive success (Bonier et al., 2009; Breuner et al., 2008; Clutton-Brock, 2016; Cowlishaw and Dunbar, 1991), studies that investigated the proximate mechanisms by which male social rank and physiological stress are responsible for inter-individual variation in reproductive success are currently lacking (Breuner et al., 2008; Cavigelli and Caruso, 2015; Dantzer et al., 2016; Moore and Hopkins, 2009).

Studies in behavioural ecology and socio-endocrinology that aim to investigate the causes and consequences of variation in physiological stress typically assess relative differences in hormone concentrations between individuals, environments, and life-history stages (see Chapter 5; see also Akinyi et al., 2017; Goymann et al., 2001; Markham et al., 2014; Seltmann et al., 2017). Such studies require large sample sizes to allow the inclusion of numerous covariates in statistical models and to reach sufficient statistical power. They further rely on enzyme immunoassays that detect small differences in hormones concentrations (that is, immunoassays of high analytical sensitivity) and that provide comparable measurements (that is, immunoassays with a stable accuracy; Wild, 2013).

6.2.1 Ensuring measurement comparability for non-invasive studies of physiological stress

The accuracy of enzyme immunoassays designed for the quantification of hormone metabolite concentrations in faecal and urine samples is known to be prone to fluctuation as a result of modifications in the analytical protocol and variation in environmental conditions in the laboratory (Noble et al., 2008; Palme, 2019; Wasser et al., 2010; Watson et al., 2013). In Chapter 4, I presented a concrete case of dramatic variation in the accuracy of an enzyme immunoassay designed to quantify cortisol metabolite concentrations in faeces of spotted hyenas. I illustrated how differences in the accuracy of the enzyme immunoassay between batches of laboratory analyses can affect the comparability of measurements and impair the validity of the interpretations.

When analytical control parameters indicate significant changes in accuracy, common practice to ensure the comparability of measurements is to (re-)assay all samples together and within a short period of time (Wild, 2013). Such an approach, however, is costly in time, manpower, and finances, and is not applicable when samples have been depleted. In Chapter 4, I developed a novel method to improve the applicability of enzyme immunoassays for research projects dealing with large data sets, as is typically the case for long-term and longitudinal studies. The method restores the comparability of measurements for all samples by using a standardisation formula fitted on a small subset – less than a quarter of the complete sample set – of re-assayed samples. To determine the size of the required subset and retrieve the standardisation formula, I used over four hundred faecal samples to model the relationship between their concentrations as initially measured and their matched re-measured concentrations. I performed simulations on decreasing numbers of samples to determine the smallest subset that provided a reliable standardisation formula (that is, a formula that generated standardised concentrations that differed from their re-measured concentrations by a coefficient of variation ≤ 20%).

The method developed in Chapter 4 constitutes an effective and reliable alternative to the re-assaying of complete sample sets. Because the method incorporates concepts and criteria from the field of analytical endocrinology (for example, coefficient of variation ≤20%; Behie et al., 2010; Goymann et al., 1999; Wild, 2013) into statistical procedures, it provides a set of intuitive and flexible tools. The method thereby can easily be applied by scientists working with other immunoassays, sample matrices, hormones or model species. The method is particularly useful for collaborative projects that share the laboratory workload between different facilities and are likely to experience variation in assay performance and accuracy. It is also valuable for long-term and longitudinal studies that typically deal with large data sets and may not be able to re-assay all samples whenever new samples are collected or a new research question is investigated. Moreover, it can be applied to standardise measurements of samples that are no longer available. This can significantly increase sample sizes, enhance the power of statistical analyses and allow the inclusion of a larger number of covariates in statistical models, which may be important for a better understanding of complex ecological and social processes.

6.2.2 Physiological stress as mediator of rank-related reproductive skew

In Chapter 5, I investigated the role of physiological stress and physiological constraints as proximate mediators of rank-related variation in reproductive performance and success among males. I specifically tested whether (i) social and sexual activities involving interactions between males influenced male physiological stress, (ii) male rank-related dominance potential influenced physiological stress in these contexts, and (iii) rank-related differences in physiological stress impacted male sociality, reproductive investment and how males allocated their reproductive effort among females of different reproductive value. The findings were consistent with the hypothesis that rank-related physiological stress arising from differences in dominance potential is a key proximate mediator of variation in reproductive investment and hence reproductive success in male spotted hyenas.

6.2.2.1 Male dominance potential influences physiological stress

I found that male physiological stress differed between high-ranking and low-ranking males only in contexts where males could exploit their dominance potential and where differences in dominance potential mattered. Low-ranking males, with relatively low dominance potential, had higher physiological stress than high-ranking males when they engaged in social activities with other males and when courting a female and concurrently competing with other males. In contrast, physiological stress was similar for males of all social ranks when they did not interact with other males, that is, when alone and when courting a female in the absence of male competitors. These results show that intrasexual interactions incur higher physiological costs for low-ranking males and suggest that low-ranking males may experience stronger physiological constraints than high-ranking males.

6.2.2.2 Rank-related physiological stress influences male performance

The study in Chapter 5 demonstrated that high-ranking and low-ranking males differed in their allocation of time to social activities, sexual activities and self-maintenance. I found that low-ranking males invested less than high-ranking males in activities that elicited comparatively high physiological stress; they spent more time alone, less time engaging in social and sexual activities and they invested less in the most attractive and most contested females – that is, females of high social rank – than did high-ranking males. I further found that male physiological stress was lowest when they spent time alone, demonstrating that staying away from clan members is an effective strategy in fission-fusion societies to downregulate physiological stress (Aureli et al., 2008; Smith et al., 2008). These tactics constitute an effective behavioural response by low-ranking males to avoid physiologically costly intrasexual competition and downregulate their physiological stress to levels similar to those of high-ranking males – as indicated by the lack of an overall correlation between physiological stress and male social rank – (see also Goymann et al., 2003). Reducing reproductive investment and focusing the investment on females of lower social rank also reduces their chance to be chosen as mates and to mate with females of high reproductive value (East et al., 2003; Szykman et al., 2001). Consistent with these findings, the study in Chapter 3 showed that the reproductive success of low-ranking males is both quantitatively and qualitatively lower than that of high-ranking males. The studies of Chapter 3 and Chapter 5 thereby provide strong support for the hypothesis that rank-related physiological constraints play a key role in shaping patterns of reproductive skew among male spotted hyenas.

6.2.2.3 Social constraints influence male performance

The findings in Chapter 5 further suggested that differences in male performance and reproductive success were the result of behavioural trade-offs shaped by the combination of physiological and social constraints. In spotted hyenas and most other group-living mammals, investment in social activities is essential for the social integration and reproductive prospects of males (Berghänel et al., 2011; East and Hofer, 2001; Gilby et al., 2013; Ryder et al., 2009; Smith et al., 2010; Vullioud et al., 2019; Willisch et al., 2010; Wiszniewski et al., 2012; Young et al., 2013). The time and physiological resources that males allocate to social activities are predicted to reduce their ability to invest in sexual activities. The study in Chapter 5 revealed that all males allocated most of their time to social activities, including low-ranking males for which social interactions incurred comparatively high physiological costs. In line with the prediction, male investment in reproduction was constrained by the time and physiological resources that males allocated to social activities. Philopatric males who are socially well integrated (Ilany et al., 2015; Vullioud et al., 2019) spent less time engaging in social activities and more time engaging in sexual activities than immigrant males. Similarly, high-ranking immigrant males who have become well integrated in the clan, experienced lower physiological stress when engaging in social activities, spent less time alone and more time engaging in sexual activities than low-ranking males. Thus, as predicted by studies about the importance of social and physiological constraints and trade-offs (Dunbar et al., 2009; Pollard and Blumstein, 2008; Zera and Harshman, 2001), the comparatively low physiological and social constraints experienced by high-ranking immigrant males and philopatric males enabled them to allocate more time and physiological resources to fitness-enhancing social and sexual activities.

6.2.2.4 Possible influence of seasonal energetic constraints

Glucocorticoids are involved in the regulation of, and in trade-offs with, a broad range of metabolic, physiological, and immunological functions (Hau et al., 2016; Ricklefs and Wikelski, 2002; Romero et al., 2009). Free-ranging individuals should be able to adaptively adjust their physiology and behaviour to a range of predictable environmental and social contexts. However, if superimposed, unpredictable challenges occur, such as a disease outbreak or a dramatic food shortage, the capacity of the individual to respond adaptively, through physiological and behavioural adjustments, may be limited and insufficient (Goymann and Wingfield, 2004; Koolhaas et al., 2011; MacLarnon et al., 2015; Romero et al., 2009). The physiological mechanism proposed in Chapter 5 to explain the relationship between male rank-related dominance potential and physiological constraints, could explain deferential allocation of resources to immunity by individuals of different social rank, and why low-ranking individuals often are more likely than high-ranking individuals to show external signs of infection or die during a disease outbreak even if they have a lower contact rate to pathogen sources than high-ranking animals (East et al., 2001; Höner et al., 2012; Marescot et al., 2018).

Seasonal variation in local food abundance can also impose additional constraints on the physiology and behaviour of animal (MacLarnon et al., 2015). In ecosystems such as the Serengeti, large herbivores undertake large-scale migrations and local prey density can drop dramatically and remain low over extended periods of time. In this ecosystem, spotted hyenas undertake long-distance, extra-territorial excursions or “commuting trips” of up to 100 km away from their clan territory to forage on herds of migratory prey (Hofer and East, 1993a). In addition to being energetically costly, these excursions are likely to incur substantial physiological costs owing to increased risks of being the target of aggression by unfamiliar resident clan members (Goymann et al., 2001; Hofer and East, 1993b). Investing in sexual activities while undertaking these excursions may be energetically and physiologically costly to males. Based on the findings in Chapter 5, I would predict that male spotted hyenas in the Serengeti ecosystem adjust their behaviour during periods of local prey scarcity to reduce these costs; they should invest less in sexual activities and/or chiefly focus their reproductive investment on high-ranking females – who are least likely to undertake long-distance excursions (Hofer and East, 2003) – or on females with older cubs not stationed at the communal den any more – who are less likely to undertake frequent commuting trips (Hofer and East, 1993a). Although there is currently no published physiological data on males, movement patterns recorded from radio-collared individuals are consistent with these predictions: clan members usually travel alone, and the commuting pattern of males is similar to that of ‘non-denning’ females, suggesting that males may reduce their reproductive investment during excursions and may not sexually invest in courting females that are likely to do frequent and costly commuting trips back to the clan territory (Hofer and East, 1993a, 1993b).

6.2.2.5 The importance of context-dependent physiological stress and behavioural adjustments

A previous study formalised the relationship between physiological stress and the competitive regime and availability of coping outlets in group-living species, and built a framework to derive predictions on the physiological costs borne by dominant and subordinate individuals of a social group (Goymann and Wingfield, 2004). This framework predicts that in societies such as that of the spotted hyena where dominance relationships are formalised by social conventions (East and Hofer, 2001; Foerster et al., 2016; Holekamp and Smale, 1991), high-ranking and low-ranking individuals should experience similar physiological costs and constraints and have similar overall physiological stress. This prediction was derived from the hypothesis that in convention-based societies, dominance relationships are stable and thus predictable, and acquiring and maintaining a social rank usually does not involve high rates of aggression and should thereby incur relatively low physiological costs for both dominant and subordinate males (Goymann and Wingfield, 2004; Sapolsky, 2005). Only during times of social instability would physiological stress be expected to be increased, as was the case in female Serengeti hyenas during times of social instability (Goymann et al., 2001). The lack of a significant association between male social rank and overall physiological stress found in Chapter 5 appears consistent with this prediction and suggests that high-ranking and low-ranking males bear similar physiological costs (or “allostatic load”).

In Chapter 5 I demonstrated that high-ranking and low-ranking males experienced different physiological costs when engaging in social and sexual activities and that the lack of an association between rank and physiological stress resulted from behavioural adjustments by the males to their different physiological constraints rather than the males having similar physiological constraints in all contexts. I further showed that the behavioural adjustments by low-ranking males incur substantial reproductive costs in terms of reduced reproductive rates and quality of offspring sired (see Chapter 3). These findings thereby highlight the importance of considering the social and sexual context and the dominance potential of males in these contexts (see also Markham et al., 2014; Sapolsky and Ray, 1989; Setchell et al., 2010; Wolf et al., 2018), when investigating rank-related physiological stress and its consequences on the performance and reproductive success of individuals.

6.2.2.6 Implications for studies on reproductive skew

The study in Chapter 5 provides strong evidence that male dominance potential mediates the physiological costs of intrasexual competition and influences the male's ability to socialise, invest in reproduction and have access to mates of high reproductive value. The study thereby shows that physiological constraints play a pivotal role in the emergence of rank-related male reproductive investment and reproductive skew in group-living species. The study also demonstrates that rank-related dominance potential and physiological constraints underpin differences in competitive ability and reproductive performance among males of different social rank. The study thereby provides an alternative mechanism to the conventional idea which posits that differences in fighting ability and physical attributes among males are responsible for differences in reproductive success, and has the potential to explain the emergence of rank-related reproductive skew in group-living species independent of the competitive regime among males.

The study also provides insights into the influence of variation in the social organisation, breeding system, and fission-fusion dynamics of group members in shaping rank-related physiological constraints and social constraints, and how these may account for the different patterns of reproductive skew within and between species. The study further provides insights into the emergence of alternative reproductive tactics (Taborsky et al., 2008) among males of different social rank whereby dominant males invest in successful, physiologically potentially costly sexual behaviours (for example, mate-guarding) whereas subordinates either forgo reproduction, or adopt reproductive behaviours that circumvent the physiological costs of intrasexual competition within the group, such as opportunistic sneaky copulation, male-male coalitions, or extra-group copulation (Corlatti et al., 2012; Gesquiere et al., 2011; Isvaran and Clutton-Brock, 2007; Young et al., 2007).

6.3 General conclusion

By showing that the distribution of potential breeding females across natal and nonnatal clans is the main driver of dispersal patterns in male spotted hyenas, the thesis provides novel insights into the processes leading to the coexistence of philopatry and dispersal within the same sex of a species. The thesis also increases our understanding of the evolution of dispersal by showing that philopatry and dispersal can be the result of a single process of breeding-group selection and by demonstrating that philopatric males and dispersers can have similar reproductive success.

By showing that social rank is a strong determinant of reproductive success in male spotted hyenas, the thesis demonstrates that high-ranking males can influence the sexual investment and reproductive performance of lower-ranking males even in species in which male contest competition for access to females is low, females exercise strong mate choice, and male ability to coerce females into mating is limited. Because social status in hyena society is driven by the amount of social support for each group member, the results expand our understanding of the importance of social integration and alliances in group-living species.

The thesis provides a novel method to improve the applicability of enzyme immunoassays for research projects dealing with large, longitudinal data sets. The method restores the comparability of measurements for all samples when assay accuracy varies by using a standardisation formula fitted on a small subset of re-assayed samples. It can easily be applied to other immunoassays, sample matrices, hormones, and species.

The thesis also increases our understanding of the role of physiological and social constraints and an individual’s dominance potential for reproductive investment and reproductive skew in group-living species. It shows that physiological and social constraints arising from differences in dominance potential can shape behavioural trade-offs between the allocation of time and physiological resources to social integration, reproduction, and self-maintenance and that these trade-offs cause differences in male performance and reproductive success

Overall, the findings should substantially contribute to the development of more realistic theoretical models on dispersal and reproductive skew, in particular for mammals living in multimale, multifemale social groups.

6.4 References

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