

Trade-offs between reproduction and health in free-ranging African striped mice

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Abstract Energy is limited and must be allocated among competing life-history traits. Reproduction is considered one of the most energetically demanding life-history stages. Therefore, the amount of energy an individual invests in reproduction might carry fitness costs through reduced energy allocation to other activities such as health maintenance. We investigated whether reproduction impacts health in the seasonally breeding African striped mouse (*Rhabdomys pumilio*). We measured health in individuals that reproduced (breeders) and individuals that did not reproduce (their adult offspring) and tested whether: (1) breeders' health before reproduction was similar to that of their offspring (representing a baseline); (2) breeders' health deteriorated after reproduction; (3) breeders' health after reproduction was worse than that of their offspring. We collected blood samples from 12 breeding females and 11 breeding males both at the onset and at the end of the breeding season and from 12 adult daughters and 11 adult sons that did not reproduce at the end

of the breeding season. Health was assessed using serum biochemistry analysis with VetScan Abaxis. Breeders differed considerably in their health before and after reproduction, particularly in parameters associated with digestion (lower amylase in males), metabolism (lower albumin, alkaline phosphatase, creatinine and glucose), osmoregulation (lower potassium and phosphorous in females) and immunity (higher globulin and altered alanine aminotransferase). Our results suggest that with the onset of breeding striped mice shifted their energy allocation from maintaining health to reproduction, indicating that investment into reproduction carries significant health costs.

Keywords Alternative reproductive tactics · Energetics · Homeostasis · Pathology · Stress

Introduction

Reproduction is considered to be one of the most energetically demanding life-history stages (Welcker et al. 2015), and investing in reproduction might impair an individual's health. During reproduction, individuals of both sexes might engage in energetically costly activities, such as territory defence, intrasexual contests, courtship and mate guarding (Kenagy 1987; Kotiaho et al. 1998; Sharick et al. 2015). For mammals, additional energetic costs arise during pregnancy, lactation and when caring for the offspring (Craft 1970; Künkele and Trillmich 1997; Speakman 2008; Naya et al. 2008). However, energy is a limited resource. Therefore, to maximize fitness, organisms must distribute energy among competing life-history traits, such as growth, reproduction, health and survival (Stearns 1992; Burton et al. 2011; Welcker et al. 2015). Trade-offs among these traits mean that

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variation in the rate at which energy is allocated to one trait will directly constrain other traits, influencing net fitness (Bacigalupe and Bozinovic 2002; Speakman 2008; Burton et al. 2011).

The maintenance of an individual's health centres on a well-functioning immune system, which is costly to maintain (Råberg et al. 2009). Nutrients and energy necessary for immunity have to be reallocated from other important functions (Sheldon and Verhulst 1996; Råberg et al. 1998; Martin II et al. 2002). Added challenges to the immune system, such as those experienced during an infection, can influence energy expenditure (Ots et al. 2001; Freitak et al. 2003; Careau et al. 2010). Therefore, breeding individuals might be confronted with metabolic trade-offs between the allocation of energy to the maintenance of their health and to energy-demanding activities of reproduction, such as milk production and parental care (Kenagy et al. 1989; Lochmiller and Deerenberg 2000; Råberg et al. 2002; Hegemann et al. 2012). It would be expected, therefore, that increased energetic demands associated with reproduction will be accompanied with a concomitant decrease in an individual's health. Yet, the association between health, energy expenditure and reproduction has rarely been studied (Ksiazek et al. 2014).

Organs such as the kidneys and the liver are important in maintaining homeostasis and their condition is, therefore, expected to be a particular good indicator of an individual's health status. The liver performs many essential functions related to digestion, metabolism and immunity, while the kidneys eliminate metabolic waste, regulate water homeostasis and maintain blood pH. Thus, how an individual maintains homeostasis in the face of increased energetic demands may depend on how well these two key organs function. Serum metabolites are the products of intermediary metabolism and reflect the physiological state of an animal (Artacho et al. 2007). Several serum metabolites, specific to the liver and kidneys, have been shown to change when the health of an individual is compromised. These include parameters associated with nutrition and digestion (e.g. amylase, calcium and total proteins), metabolism (e.g. albumin, alkaline phosphatase, blood urea nitrogen, creatinine and glucose), osmoregulation (e.g. potassium) and immunity (e.g. globulin), all of which have been shown to change when the body uses more energy than it can gain, as for example during periods of starvation, intensive physical exercise or reproduction (Schroeder 1984; Firmansyah et al. 1989; McBride and Kelly 1990; Gonzales et al. 2003; Rodríguez et al. 2005; Artacho et al. 2007; Tavares-Dias et al. 2009). Serum metabolites change have thus been widely used in both human and wildlife medicine to assess liver and kidney conditions and ascertain an individual's health (Payne and Payne 1987; Christopher et al. 1999; Marchal et al. 2012).

In the present study, we investigated the health costs of reproduction in a population of African striped mice (*Rhabdomys pumilio*) from the semi-arid Succulent Karoo. Striped mice are seasonal breeders, with the bulk of reproduction taking place at the height of the plant growing season in spring (July–November; Schradin 2005). During the breeding season and under conditions of high population density, striped mice live in extended family groups (Schradin et al. 2010; Schoepf and Schradin 2012). Groups consist of a single breeding male, 1–4 closely related communally breeding females and their non-reproducing male and female philopatric offspring, which remain in their natal group long after reaching adulthood (Schradin and Pillay 2004; Schradin et al. 2009a, b). Breeding females within social groups typically reproduce synchronously and rear their offspring together in a communal nest (Schradin and Pillay 2004). After a gestation period of 23 days, each female striped mouse produces a litter of approximately 5 pups 2–3 times per breeding season (Brooks 1982; Schradin and Pillay 2005).

In our study, we used serum biochemical analysis to investigate whether reproduction resulted in reduced health in breeding striped mice. Specifically, we tested: whether: (1) breeders' health before reproduction was similar to that of their non-reproducing adult offspring (comparisons between breeders at the onset of the breeding season and their offspring towards the end of the breeding season); (2) breeders' health deteriorated after they reproduced (comparisons of breeders before and after reproduction); (3) breeders' health after reproduction was worse than that of their non-reproducing adult offspring (comparisons between breeders and their offspring towards the end of the breeding season). Non-breeders were individuals born at the start of the breeding season, and reached adulthood by its end. In nature, non-breeders at the end of the breeding season can be expected to have the best health and thus represent a good baseline comparison for breeders health at the start of the breeding season. Such a comparison was thus important to show whether breeders were healthy at the start of the breeding season.

Materials and methods

Study area and field techniques

Data were collected during 2013 in Goegap Nature Reserve, South Africa. Data from breeding individuals before they reproduced were collected in the moist season (June–July), when food is abundant, rainfall is high and individuals have yet to start breeding. Data from breeding individuals after they reproduced and from their non-reproducing adult offspring were collected between mid-November and

mid-January, when food was declining, rainfall was low and after individuals had given birth to the last litter for that season. Striped mice are considered adults at around 4–6 weeks of age when they reach sexual maturity and weigh more than 30 g (Schradin et al. 2009a, b). For our study, we included only individuals that weighed at least 30 g and were more than 6 weeks of age when sampled. The age of striped mice was estimated from their body mass at first trapping, using a population-specific growth curve. Trapping, behavioural observations and radio-tracking were used to identify striped mice at the field site and to determine their social tactics (breeders or non-breeders). Striped mice were trapped twice daily directly at their nest using Sherman-like metal traps (26×9×9 cm; Schradin and Pillay 2005). Each trapped individual was weighed using an electronic field scale (± 0.1 g), sexed and its reproductive status noted. All trapped mice were permanently ear-tagged (National Band and Tag Co., Newport, KY, USA) and were marked with a non-toxic hair dye (Inecto Rapido, Pinetown, South Africa), which aided with individual recognition during behavioural observations (Schradin and Pillay 2004). Behavioural observations were performed at each group's nest at peak activity times to ascertain individual affiliation to specific groups (Schradin et al. 2010). All adult breeding individuals were fitted with radio-collars (Holohil, Carp, ON, Canada; 2.5–4.4 g) and were radio-tracked at night to confirm their sleeping locations (Schradin and Pillay 2005). Radio-tracking was performed using an AOR 8000 wide range receiver (Tokyo, Japan), an H-antenna (Africa Wildlife Tracking, Pretoria, South Africa) and a global positioning system (GPS; eTrex Venture, GARMIN International, USA) with an accuracy of ± 3 m. Measures of seasonal variation in food availability were recorded once per month using plant surveys. Plants surveys were carried out using the Braun–Blanquet Method (Werger 1974) and were conducted on eight monitoring plots (2×2 m each) located at random on the field study site to measure food availability. For each plot, we recorded the number of food plants (for details see Schradin and Pillay 2005).

Blood sampling

A total of 15 groups were involved in the study. From eight groups, we sampled one breeding female, one breeding male, one philopatric female and one philopatric male. From four additional groups, we sampled a breeding female and an adult philopatric female only, and from another three groups we sampled a breeding male and an adult philopatric male only. As breeders and philopatrics belonged to the same group and were of the same sex, our data followed a paired design for between-individual comparisons, controlling for potential ecological and lineage differences between group territories that could have affected health.

Striped mice were trapped at their nests during the morning shortly after they became active (Schradin 2008b). Traps were watched from a distance of 10 m and as soon as an individual entered a trap, it was removed and anaesthetized with diethyl ether (Schradin 2008a, b). Blood was collected using the sublingual method (Heimann et al. 2009; Schoepf and Schradin 2013). We were able to obtain a blood sample of approximately 400 μ l in less than 3 min, a time short enough to prevent a stress response from influencing the blood parameters we wanted to measure (Schradin 2008a). After collection, individuals were released at the site of capture, and blood samples were taken directly to the research station where they were allowed to clot before being centrifuged. The resulting serum was stored in aliquots at -20°C .

Blood samples of individuals belonging to the same group were collected in the early morning within a few days of each other. To assess whether breeders differed in their health after reproduction (within-individual comparisons), we collected blood samples from 12 breeding females and 11 breeding males at the onset and again at the end of the breeding season. To assess whether individuals that bred differed in their health from individuals that did not breed (between-individuals comparisons), we collected blood samples from 12 breeding females and 11 breeding males at the end of the breeding season and compared these with 12 philopatric females and 11 philopatric males. Therefore, blood was drawn twice from each breeder (at the onset and at the end of the breeding season) and once from each philopatric (at the end of the breeding season).

Assessing individual's health

The different serum parameters measured and how they related to health are summarized in Table 1 of the electronic supplementary material. Striped mice general health was assessed using a VetScan VS2 Chemistry Analyzer (Abaxis Inc., Union City, CA, USA) in a laboratory at the Succulent Karoo Research Station following procedures previously used in this species (Schoepf et al. 2016). 50 μ l of blood serum were diluted with 50 μ l of 0.9% saline solution (Alcon Laboratories, South Africa). The resulting solution was transferred to a reagent disc (Rotor, Abaxis Inc., Union City, CA, USA) and inserted into the chemistry analyzer for processing. Each disc contained all the necessary components and reagents to perform a comprehensive serum biochemistry analysis of a single sample. We used the VetScan Comprehensive Diagnostic Profile Test to analyse our samples (Comprehensive Diagnostic Profile, Abaxis Inc., Union City, CA, USA), because it uses dry and liquid reagents to produce an *in vitro* quantitative detailed analysis of several serum metabolites, important in nutrition and digestion (amylase, calcium and total proteins),

Table 1 The influence of reproduction on health in African striped mice was assessed in individuals that reproduced (breeders) and individuals that did not reproduce (their adult offspring). We tested whether: (1) breeders' health worsens after reproduction; (2) breed-

ers' health after reproduction was worse than that of their offspring; (3) breeders' health before reproduction was similar to that of their offspring

Parameter	R ²	Breeders after vs. breeders before		Breeders after vs. offspring		Breeders before vs. offspring	
		t value	p value	t value	p value	t value	p value
Nutrition and digestion							
Amylase (IU/l)	0.61	-1.13 (F)	0.28 (F)	-0.53 (F)	0.60 (F)	-1.25 (F)	0.23 (F)
	0.56	-3.75 (M)	0.005 (M)	1.47 (M)	0.17 (M)	3.87 (M)	0.003 (M)
Calcium (mg/dl)	0.16	-1.52	0.14	-1.35	0.19	0.15	0.88
Total proteins (g/dl)	0.48	-0.11 (F)	0.91 (F)	-0.40 (F)	0.70 (F)	0.48 (F)	0.64 (F)
	0.13	-0.57 (M)	0.58 (M)	-1.12 (M)	0.29 (M)	-0.55 (M)	0.59 (M)
Metabolism							
Albumin (g/dl)	0.36	-5.27	<0.0001	-2.20	0.03	2.69	0.01
Alkaline phosphatase (IU/l)	0.45	-4.90	0.0001	-3.95	0.0004	0.23	0.82
Blood urea nitrogen (mg/dl)	0.05	0.15	0.88	-1.63	0.11	-1.78	0.08
Creatinine (mg/dl)	0.16	-2.47	0.02	3.06	0.005	3.35	0.002
Glucose (mg/dl)	0.51	-5.45	<0.0001	-2.03	0.05	2.71	0.01
Ketones (mmol/l)	0.47	2.01	0.06	-2.81	0.01	-6.95	<0.0001
Osmoregulation							
Phosphorous (mg/dl)	0.21	-2.59 (F)	0.03 (F)	1.00 (F)	0.34 (F)	2.51 (F)	0.03 (F)
	0.61	1.42 (M)	0.20 (M)	3.84 (M)	0.003 (M)	-3.56 (M)	0.005 (M)
Potassium (mmol/l)	0.54	-4.60	0.0002	-2.61	0.01	0.11	0.91
Sodium (mmol/l)	0.01	-0.18	0.86	-0.82	0.42	-0.64	0.53
Damage and immunity							
Alanine aminotransferase (IU/l)	0.63	2.37 (F)	0.049 (F)	3.71 (F)	0.003 (F)	-1.22 (F)	0.23 (F)
	0.34	-2.83 (M)	0.02 (M)	-2.65 (M)	0.02 (M)	3.21 (M)	0.01 (M)
Globulin (g/dl)	0.40	4.66	0.0001	3.85	0.0005	-0.29	0.77
Total bilirubin (mg/dl)	0.32	0.43	0.67	3.37	0.002	2.98	0.005
Body mass (g)	0.91	0.58 (F)	0.57 (F)	7.33 (F)	<0.0001 (F)	7.00 (F)	<0.0001 (F)
	0.95	5.25 (M)	0.0004 (M)	11.46 (M)	<0.0001 (M)	8.67 (M)	<0.0001 (M)

If reproduction imposes health costs we expected breeders to be in poorer health after reproducing and when compared to their offspring. Numbers in bold represent significant values.

metabolism (albumin, alkaline phosphatase, blood urea nitrogen, creatinine and glucose), osmoregulation (phosphorous, potassium and sodium), damage and immunity (alanine aminotransferase, globulin and total bilirubin) (see Supplementary Material: Table 1 for a detailed description of the parameters measured and their functions). This profile is widely used by veterinarians to determine an animal's general health and diagnose potential malfunctioning

of the liver and the kidneys. Sample results were transferred to a computer using Eltima Software (Eltima Software GmbH, Frankfurt, Germany). Because ketone measurements were not included in the VetScan Comprehensive Diagnostic Profile Test, but are an important indicator of lipid metabolism, they were obtained separately during blood sampling in the field using a digital ketone analyser (FreeStyle Optimum, Abbott Diabetes Care Ltd., Oxon,

UK). The first drop of blood obtained after an individual had been sampled (see above) was collected onto a ketone strip and was inserted into the analyzer to obtain instant data (Schoepf et al. 2016).

Data analysis

Data analysis was performed using R version 3.2.4 (The R Foundation for Statistical Computing, Vienna, Austria). We checked for the normal distribution of the data using the Shapiro–Wilk test. Albumin, alkaline phosphatase, alanine aminotransferase, amylase, body mass, blood urea nitrogen, calcium, creatinine, globulin, glucose, ketones, phosphorous, potassium, sodium and total proteins were log-transformed to reach normality. We used linear mixed effects models (LMMs; Package lme4; Bates et al. 2014) to measure whether: (1) breeders' health worsens after reproducing; (2) breeders' health after reproduction was worse than that of their non-reproducing adult offspring; (3) breeders' health before reproduction was similar to that of their non-reproducing adult offspring. Each LMM had either body mass or one of the serum biochemistry parameters as the response variable while breeding status (breeder, philopatric) was the fixed factor. As several serum metabolites have been shown to vary with age, availability of food resources, and according to the sex of an individual (Barnes et al. 2008), age (weeks), and food availability (mean number of food plants available/month) were entered as additional covariates, while sex (male, female) was entered as a co-factor in each model. In addition, because the costs of (allo-)parental care depends on the number of offspring taken care of (see Speakman 2008 and references therein), the number of offspring cared for by a breeder was also included as an additional co-variate in each LMM. In African striped mice, breeders of both sexes participate in costly parental care (Schradin and Pillay 2005), and females also show allo-parental care including allo-nursing towards offspring of other females (birth are typically synchronized; Schradin and Pillay 2004; Schradin et al. 2009c); therefore, for each individual we calculated the total number of offspring weaned in the group during an individual's tenure as breeder. Co-factors and co-variate effects were assessed using likelihood ratio tests by comparing models with and without each co-factor, while maintaining the same random effects structure (Zuur et al. 2009). Individual ID nested within Group ID was entered as a random factor in each model. We selected the model that best fitted our data using stepwise backward procedures between saturated models (including covariates interactions) and simple models (excluding covariates interactions) (Crawley 2007). For all models, we report the R^2 (adjusted) as calculated following Nakagawa and Schielzeth (2013). We verified our model selection by (1) plotting the model residuals vs. the fitted values, (2) checking the normal distribution of the model

residuals using normal probability plots, (3) checking for heteroscedasticity, and (4) leverage (Crawley 2007). In addition, we used a multivariate analysis of variance with Wilks criterion (MANOVA, library MASS) on all health parameters collected for breeders to verify group-specific profiles variation before and after reproduction took place. We used linear discriminant analysis (LDA, library MASS; Venables and Ripley 2002) on the scaled down MANOVA parameters to assess the extent of the disparity in health parameters between breeders before and after they reproduced and thus to assess an overall health index for reproduction. Two re-sampling methods, a jack-knifed classification (leave-one-out cross-validation) and a randomized sample validation were used to assess the validity of the LDA (McGarigal et al. 2000). We calculated Pearson's Chi-squared test with Yates' continuity correction to assess whether the cross-validation method was a better fit than the random method. The LDA model that best fitted our data was used to produce a histogram of discriminant scores, graphically displaying the ability of the discriminant function to separate before from after reproduction. The factor with the highest LDA loading was taken to be a good predictor of health for that category. All data used were z-transformed prior to factor analysis. All tests were two tailed. For all tests, a significance level (α) of 0.05 was selected.

Results

Sex differences

Including sex as a co-factor improved model fit for amylase, total proteins, phosphorous, alanine aminotransferase and body mass (Supplementary Material: Table 2), indicating that males and females significantly differed in these parameters. Therefore, all subsequent analysis involving these factors was run separately for the two sexes.

Age effects

Including age as a co-variate improved model fit for potassium and male phosphorous and female alanine aminotransferase, indicating that there was a significant effect of age for these parameters (Supplementary Material: Table 3). Specifically, we found that older individuals had lower potassium ($t=-2.12$, p value=0.046) than younger individuals, males had higher phosphorous than younger males ($t=3.14$, p value=0.02) and older females had higher alanine aminotransferase than younger females ($t=2.66$, p value=0.03).

The effects of seasonal variation in food availability

Including the number of food plants available as a co-variate improved model fit for creatinine, female phosphorous and female and male alanine aminotransferase, indicating that these parameters were significantly affected by the seasonal variation in food availability (Supplementary Material: Table 4). Specifically, we found that individuals had higher creatinine ($t=3.35$, p value=0.002), females had higher phosphorous ($t=2.51$, p value=0.03) and lower alanine aminotransferase ($t=-3.31$, p value=0.01), and males had higher alanine aminotransferase ($t=3.58$, p value=0.01) when food was more abundant at the onset of the breeding season.

The effects of numbers of offspring weaned

Including the number of offspring cared for by a breeder as a co-variate improved model fit for ketones and male amylase (Supplementary Material: Table 5). Specifically, we found that individuals that had weaned more offspring had higher ketones ($t=3.03$, p value=0.01) and males tended to have higher amylase ($t=2.21$, p value=0.05).

Interactive effects

The interaction between number of offspring weaned and age positively affected male phosphorous and female alanine aminotransferase (Supplementary Material: Table 6). Older males that had cared for more offspring had higher phosphorous ($t=3.28$, p value=0.02), while older females that had weaned more offspring had higher alanine aminotransferase ($t=4.00$, p value=0.005). The interaction between age and the number of food plants available positively affected male phosphorous (Supplementary Material: Table 6). Specifically, we found that older males had higher phosphorous when food was less abundant at the end of the breeding season ($t=3.19$, p value=0.02). The interaction between the number of offspring weaned and the number of food plants available positively affected female alanine aminotransferase (Supplementary Material: Table 6). Specifically, we found that females that had weaned more offspring had higher alanine aminotransferase when food was less abundant at the end of the breeding season ($t=4.82$, p value=0.002).

Was breeders' health before reproduction similar to that of their non-reproducing adult offspring?

Paired comparisons between breeding individuals before they reproduced and their non-reproducing philopatric offspring showed that breeding individuals had lower ketones (Fig. 2e) and lower phosphorous (males only;

Fig. 3a), but higher amylase (males only; Fig. 1), higher albumin (Fig. 2a), higher creatinine (Fig. 2b), higher glucose (Fig. 3c), higher phosphorous (females only; Fig. 3a), higher alanine aminotransferase (males only; Fig. 4a) and higher total bilirubin levels (Fig. 4c), and were heavier (females and males; Fig. 5) than their non-reproducing philopatric offspring (Table 1).

Was breeders' health after reproduction worse than before reproduction?

Paired comparisons of individuals before and after reproduction showed that at the end of the breeding season, breeders had significantly lower amylase (males only; Fig. 1), lower albumin (Fig. 2a), lower alkaline phosphatase (Fig. 2b), lower creatinine (Fig. 2c), lower glucose (Fig. 2d), lower phosphorous (females; Fig. 3a), lower potassium (Fig. 3b) and lower alanine aminotransferase (males only; Fig. 4a), but higher alanine aminotransferase (Fig. 4a), higher globulin levels (Fig. 4b) and were heavier (males only; Fig. 5) than at the onset of the breeding season (Table 1).

Was breeders' health after reproduction worse than that of their non-reproducing adult offspring?

Paired comparisons between individuals that reproduced and their non-reproducing philopatric offspring at the end

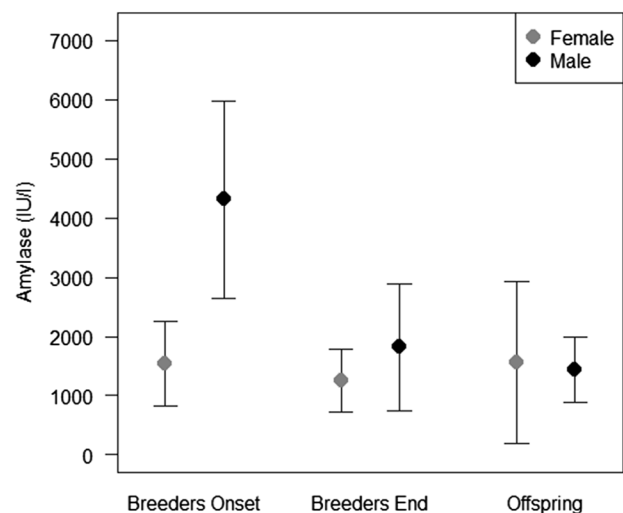


Fig. 1 Male amylase was the only parameter related to nutrition and digestion that was found to be significantly different in breeding individuals before and after reproduction and when compared to their non-reproducing adult offspring. Mean values along with their confidence ranges are reported

Fig. 2 Parameters related to metabolism in breeding individuals before and after reproduction and in their non-reproducing adult offspring. **a** Albumin, **b** alkaline phosphatase, **c** creatinine, **d** glucose and **e** ketones. Mean values along with their confidence ranges are reported

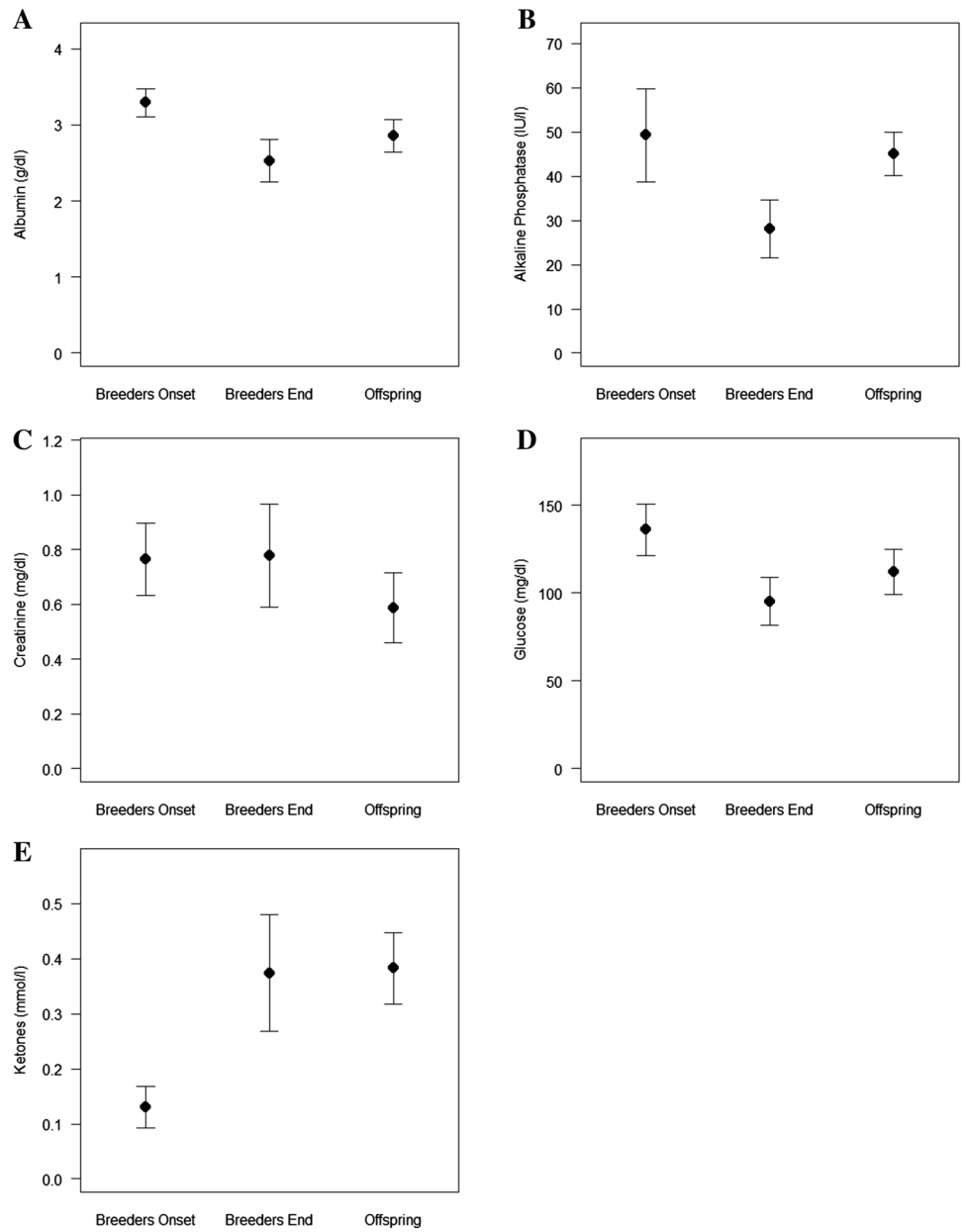


Fig. 3 Parameters related to osmoregulation in breeding individuals before and after reproduction and in their non-reproducing adult offspring. **a** Phosphorous (both sexes) and **b** potassium. Mean values along with their confidence ranges are reported

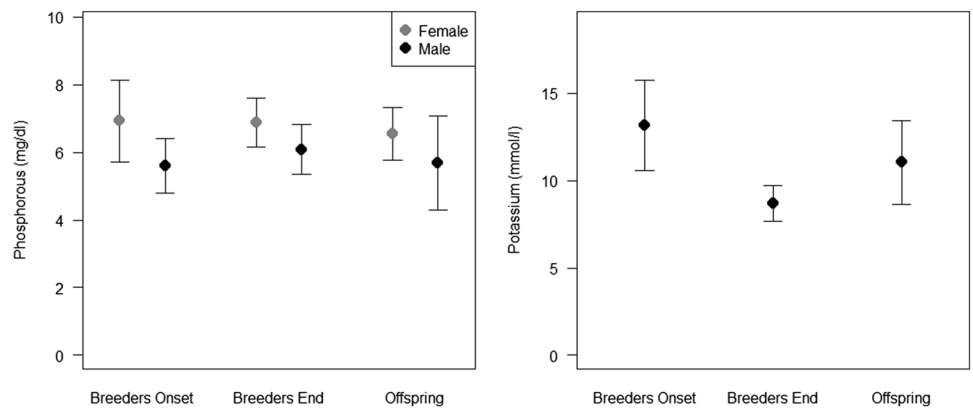


Fig. 4 Parameters related to damage and immunity in breeding individuals before and after reproduction and in their non-reproducing adult offspring. **a** Alanine aminotransferase (both sexes), **b** globulin and **c** total bilirubin. Mean values along with their confidence ranges are reported

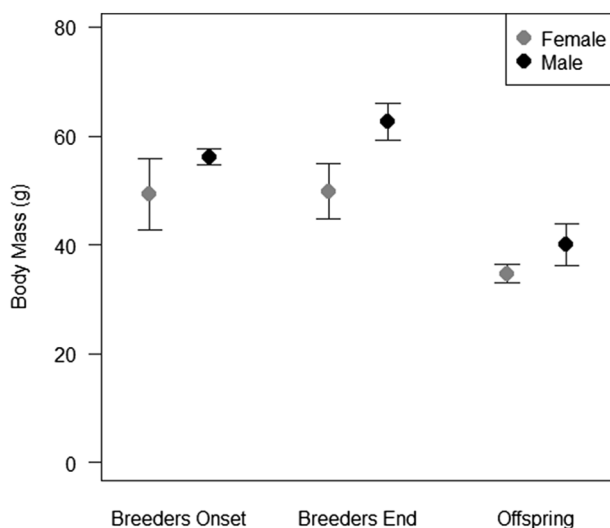
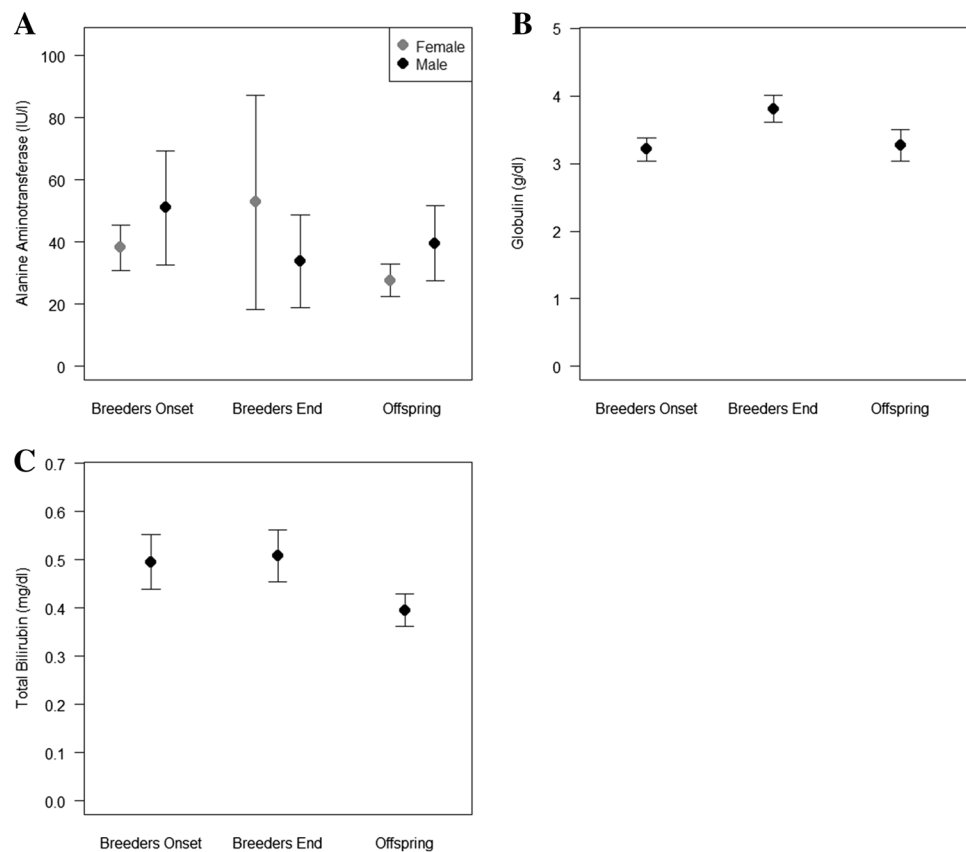


Fig. 5 Breeding individuals of both sexes were significantly heavier than their non-reproducing adult offspring both before and after reproducing. However, only breeding males were found to increase their body mass after reproducing. Mean values along with their confidence ranges are reported

of the breeding season showed that breeders had lower albumin (Fig. 2a), lower alkaline phosphatase (Fig. 2b), lower ketones (Fig. 2e), lower potassium (Fig. 3b), and

lower alanine aminotransferase (males only; Fig. 4a), but higher creatinine (Fig. 2c), higher phosphorous (males only; Fig. 3a), higher alanine aminotransferase (females only; Fig. 4a), higher globulin (Fig. 4b), higher total bilirubin levels (Fig. 4c) and were heavier (both sexes; Fig. 5a, b) than their offspring (Table 1).

Which factors were good indicators of changes in health?

MANOVA analysis indicated significant differences in breeding individuals before and after reproduction (MANOVA, Wilks' $\lambda=0.24$, $F=5.79$, $df=29$, p value <0.0001), particularly in albumin ($F=25.49$, p value <0.0001), alkaline phosphatase ($F=13.29$, p value $=0.0007$), amylase ($F=6.09$, p value $=0.02$), glucose ($F=18.05$, p value $=0.0001$), potassium ($F=10.62$, p value $=0.002$), globulin ($F=21.90$, p value $=<0.0001$) and ketones ($F=24.15$, p value <0.0001). LDA based on the jack-knifed classification matrix returned an overall 84.78% correct classification (82.61% correct for breeders before reproduction and 86.96% for breeders after reproduction). LDA based on randomized sample validation returned an overall 93.48% correct classification (95.65% correct for breeders before reproduction and 91.30% for breeders after reproduction). LDA with the jack-knifed classification did

not result in an overall increase in predictive power and, therefore, the LDA based on the randomized sample validation was chosen as the best fit for our data (Pearson's Chi-squared test with Yates' continuity correction: $\chi^2=22.26$, $p<0.0001$). The mid-way point that allowed us to discriminate which variable belonged to which group was 0. This indicated that variables that loaded negatively on LD1 belonged to breeders before reproduction and variables that loaded positively on LD1 belonged to breeders after reproduction. Potassium, albumin and glucose loaded negatively on LD1 (-1.59 ; -0.16 , -0.02 , respectively), whereas ketones, globulin, amylase and alkaline phosphatase loaded positively on LD1 (2.04 , 1.16 , 0.36 , 0.16 , respectively). Thus, potassium and albumin were taken to be overall good health indicators for individuals before they reproduced, while ketones, globulin, amylase and alkaline phosphatase were taken to be overall good health predictors for individuals after they reproduced.

Discussion

Reproduction was associated with decreased health, as indicated by changes in several serum metabolites, despite the fact that males were heavier after reproducing and that breeders of both sexes, in general, had high body mass (when compared to adult non-breeders). Specifically, we found that breeders had lower amylase (in males), albumin, alkaline phosphatase, creatinine, glucose, phosphorous (in females), potassium, higher globulin levels and altered alanine aminotransferase (lower in males and higher in females). Overall, these results suggest that investing in reproduction carries significant health costs.

Serum metabolites that were found to change in response to reproduction were associated with metabolism, osmoregulation, damage and immunity, and additionally, for males only, nutrition and digestion. Serum parameters associated with metabolism that were found to be particularly altered in breeding individuals were glucose, ketones, albumin, alkaline phosphatase and creatinine. When glycogen stores are depleted, endotherms typically start lipid metabolism, which provides energy in the form of ketone bodies (Robin et al. 1988; McCue 2010). In our study, breeders had overall higher ketones when compared with their non-reproducing adult offspring. Ketone levels were particularly elevated in individuals that had weaned more litters. Higher ketone levels were coupled with decreased glucose levels. Glucose is the end product of carbohydrate metabolism and is the primary energy source for the body (Fischbach 2003; Van Leeuwen and Poelhuis-Leth 2009; Pagana et al. 2015). As such, glucose concentration is kept within specific limits that are critical for the brain and the central nervous system function (Rodríguez et al. 2005).

Low levels of glucose are typically observed in individuals that are using more energy than they can gain, for example, when fasting (Artacho et al. 2007; Tavares-Dias et al. 2009; McCue 2010; Mustonen et al. 2013). In striped mice glucose levels are positively correlated with food availability (Schradin et al. 2015). Interestingly, however, glucose levels in our study were not affected by the availability of food plants, indicating that the decreased glucose levels observed were due to increased energy investment in reproduction and not decreased food availability.

Decreased levels of glucose in breeders were associated with decreased albumin, alkaline phosphatase and creatinine levels. The primary role of albumin in the blood is to maintain colloidal osmotic pressure (Fischbach 2003; Hopkins 2005; Brunner 2009). However, in serum, albumin is also a measure of nutrition, particularly indicating whether sufficient proteins are absorbed by the body (Schroeder 1984; Payne and Payne 1987; Pagana et al. 2015). As albumin can be used as an energy source, after glycogen and lipid reserves are depleted (Ots et al. 1998), decreased albumin levels are typically indicative of long-term protein deficiency and malnutrition (Fischbach 2003; Gonzales et al. 2003; Hopkins 2005). Albumin levels are also decreased during pregnancy and in older individuals (Fischbach 2003; Van Leeuwen and Poelhuis-Leth 2009). Alkaline phosphatase is of diagnostic significance because of its involvement in the detoxification of endotoxins and increased levels of this enzyme are typically measured in younger individuals or in females in the advanced stages of pregnancy (Van Leeuwen and Poelhuis-Leth 2009). Studies in rodents and marine mammals have shown that alkaline phosphatase can, however, be significantly reduced in individuals experiencing a decline in food availability (Jain et al. 2013) or as a consequence of a parasitic infection (Fothergill et al. 1991; Evans 2005). In our study, albumin and alkaline phosphatase were not affected by either age or food availability, suggesting that the decrease in both of these parameters rather stemmed from the increased energetic demand associated with reproduction. Creatinine is a by-product of muscle catabolism and high creatinine concentrations typically reflect greater muscle mass (Barnes et al. 2008). Breeders of both sexes had much higher creatinine levels overall when compared with their non-reproducing adult offspring and were also much heavier. Interestingly, however, we found that creatinine levels significantly decreased in breeders after reproduction, despite the fact that body mass increased in both sexes. This would rather indicate a physiological cost and deteriorating health, not a gain in muscle mass. Creatinine is also a good indicator of protein deficiency (Artacho et al. 2007; Brunner 2009; Awerman and Romero 2010) and reduced levels of this metabolite indicate that protein intake from food decreased in breeders (also supported by the fact that creatinine was

affected by availability of food resources). Creatinine was higher when food was more abundant. Taken together, these results indicate that reproduction carried significant costs, which resulted in breeders entering a negative energy state.

Serum parameters associated with osmoregulation that were found to be altered in breeders were potassium and phosphorous (in both sexes). Potassium levels are of clinical significance because potassium is important in the functioning of the neuromuscular system, particularly the heart muscle, regulates water balance and assists in the synthesis of proteins and carbohydrates for fuel (Fischbach 2003; Hopkins 2005; Van Leeuwen and Poelhuis-Leth 2009). Potassium is typically kept within a very narrow range; therefore even small changes in potassium level can have profound effects on body functions (Hopkins 2005). Low potassium levels are typically associated with muscle weakness and starvation (Fischbach 2003; Hopkins 2005; Van Leeuwen and Poelhuis-Leth 2009). Phosphorous is required for the synthesis of several proteins and enzymes, and plays a prominent role in the intermediary metabolism of carbohydrates (Sreedhar et al. 2013). Serum phosphorous concentration is related to protein intake. Phosphorous plays an important role in intermediary cellular metabolism (Van Leeuwen and Poelhuis-Leth 2009). Previous studies in moose (*Alces alces*) found that phosphorous can be decreased in lactating individuals (Franzmann and LeResche 1978). In our study, females had significantly lower phosphorous after reproduction, indicating an energetic cost in these individuals. The lower potassium and phosphorous levels observed in breeders thus indicated that their osmoregulation was impaired. This was particularly evident in older breeders, which had the lowest potassium levels and in breeding females, which had lower phosphorous levels towards the end of the breeding season. Another important function of phosphorous is also in bones and teeth formation, as such increased phosphorous levels can indicate severe tissue trauma or bone fractures, but also dietary imbalance (Schroeder 1984; Sreedhar et al. 2013). In our study, older breeding males that had cared for more offspring had an overall higher phosphorous at the end of the breeding season, thus also suggesting costs associated with deteriorated osmoregulation in breeding males.

Serum parameters associated with organ damage and immunity that were altered in breeders were alanine aminotransferase, globulin and total bilirubin. Alanine aminotransferase is released from liver cells into the bloodstream following liver cell death resulting from hypoperfusion or toxicity (Brunner 2009). Elevated levels of alanine aminotransferase were observed in breeding females when compared to their non-reproducing offspring, with older females that had weaned the most offspring having particularly high values when food was more abundant,

and were associated with an increase in globulin levels. Globulin, which is typically not affected by diet, consists of both acute phase proteins and immunoglobulin that act in response to injuries and infections (Ots et al. 1998; Fischbach 2003; Evans 2005). Globulin forms part of the antibody structure and is a good indicator of immune system function (Ots et al. 1998). In both acute disease and chronic infection, sick individuals typically have higher globulin than healthy individuals (Ots et al. 1998). The increased globulin levels observed in breeders were possibly the result of infections arising from injuries suffered during aggressive territorial encounters with neighbouring individuals (Schradin 2004). Alternatively, breeders' ailing health could have resulted from an increase in globulin following their higher susceptibility to parasitism. Interestingly, however, and contrary to females, male alanine aminotransferase levels were lower when food was more abundant. In a previous study, we showed that alanine aminotransferase levels increase as the dry season progresses and remain elevated well into the moist season (Schoepf et al. 2016). The observed difference between males and females in this study could thus indicate that (1) males were able to downregulate their alanine aminotransferase levels and recover from the damage incurred during the dry season, thus supporting the idea that alanine aminotransferase levels can be restored back to normal levels once they have been disrupted, but take long to do so; and (2) breeding females could have taken longer to downregulate their alanine aminotransferase levels than males because of the additional costs associated with pregnancy and lactation. While total bilirubin levels of breeders did not differ between before and after they bred, values were significantly elevated compared to those of their non-reproducing offspring, further indicating that breeders' health was compromised. Elevated total bilirubin levels, in fact, typically indicate severe haemolysis resulting from the destruction of red blood cells (Kopeck and Harvey 1995; Bossart et al. 2001). Studies in black bears (*Ursus americanus*) found that total bilirubin can be significantly higher in older than younger individuals (Schroeder 1984; Barnes et al. 2008). However, in our study total bilirubin was not affected by age. Nonetheless, it is possible that the observed increase in total bilirubin could have been a consequence of cellular damage associated with both ageing and breeding, as reproduction is regarded as one of the main contributors to ageing (Rose and Bradley 1998). These results thus suggest that breeding individuals were in poorer health. In addition, we found that while breeding males had an overall higher amylase than their offspring, the levels of this metabolite significantly decreased after they reproduced, with lowest values found in older males which had cared for more offspring. This is interesting as it does not only confirm that reproduction results in significant health costs, but also

suggests that caring for offspring may require additional energy investment by breeding males.

Overall our results indicate that breeders shifted energy they had previously allocated to their health towards reproduction, which resulted in significant metabolic and osmoregulatory costs. Several studies have shown that increased reproductive effort decreases parents' chance of survival to the next breeding season or decreases offspring quality in the future (Oksanen et al. 2001; Koivula et al. 2003; Knowles et al. 2009) and declining health may underpin these costs. For example, a study in female eastern grey kangaroos (*Macropus giganteus*) found that reduced reproductive effort appeared to increase individual condition and subsequent reproductive success (Gélin et al. 2015). When resources decline, healthy individuals typically make behavioural, morphological and/or physiological adjustments to maintain homeostasis (Koolhaas et al. 2011; Jessop et al. 2013; Schulte 2014; Rymer et al. 2016). However, individuals whose health is already compromised when resources decline may be unable to make such adjustments, which will result in further health deterioration and increased risk of death. Changes in serum parameters particularly associated with metabolism (albumin, alkaline phosphatase, glucose and ketones) and immunity (globulin) during reproduction may have particular far-reaching consequences. This is the case in striped mice, where individuals that entered the dry season with decreased albumin, glucose and increased globulin were unable to adjust their resting metabolic rate during the dry season and did not survive (Schoepf et al. 2016). In our study, the difference in reproductive investment between breeders and their non-reproducing offspring might have constrained them in investing in health. On the other hand, adult non-reproducing offspring might rather invest in their health and postpone reproduction until they are in a better physiological condition to breed.

Altogether, our results indicate that reproduction carried significant health costs for breeders, which in some cases were exacerbated by ageing or reduced availability in food resources due to deteriorating environmental conditions. Blood biochemical parameters are subject to changes with increasing age (Ihrig et al. 2001; Mohri et al. 2007) and are impacted by seasonality, particularly variations in energy balance and fat storage, in a number of species (Matula et al. 1980; Perret 1998; Veloso and Bozinovic 2000; Kenagy et al. 2002; Massányi et al. 2009). Differences in these metabolites indicate that seasonal reduction in food availability and quality (reduced protein and water content) together with ageing effects were contributing factors affecting breeders' health. To our knowledge, this is the first comprehensive study to investigate the health implications of reproduction in a free living sociable mammal. Overall, our results show that reproduction does carry

health costs, but also that health is in itself a complex concept, influenced by multiple factors, which cannot be studied in isolation.

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Compliance with ethical standards

Conflict of interest None of the authors have competing interest.

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