Causes and consequences of alterations in stress physiology, immunity and oxygen delivery in a small mammalian hibernator

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Table of content

Erklärung 1

Publications and manuscripts 2

Summary 3

Chapter I: General Introduction 5

Chapter II: The leukocyte differential 17

Chapter III: The oxygen delivery system 54

Chapter IV: Stress hormones 94

Chapter V: General Discussion 124

Zusammenfassung 139

Danksagung 142

Erklärung

Eidesstattliche Versicherung gemäß § 7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat

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Ort und Datum

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Publications and manuscripts

The following publications and manuscripts are part of this thesis:

1. Havenstein, N., Langer, F., Stefanski, V., Fietz, J., 2016.
   It takes two to tango: Phagocyte and lymphocyte numbers in a small mammalian hibernator.
   Brain. Behav. Immun. 52, 71–80. doi:10.1016/j.bbi.2015.09.018
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2. Havenstein, N., Langer, F., Fietz, J., in press.
   Life History Written in Blood: Erythrocyte Parameters in a Small Hibernator, the Edible Dormouse.
   J. Comp. Physiol. B, 1-13. https://doi.org/10.1007/s00360-017-1111-8
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3. Havenstein, N., Langer, F., Weiler, U., Stefanski, V., Fietz, J.
   Building the bridge between environment, physiology and life history: stress hormones in a small mammalian hibernator.
   Manuscript in preparation for publication.

Summary

The different functions and activities of an organism require substantial amounts of energy and thus compete for the limited available resources. During demanding situations, allocation decisions potentially result in trade-offs between physiological processes that can have consequences on the performance and fitness of an individual. The investigation of these physiological trade-offs upon environmental stressors and demanding life history stages may be the key to ultimately understand mechanisms underlying the evolution of life history tactics and population declines in vertebrates. Endocrine mediators, especially glucocorticoid hormones (GCs) build the bridge between environmental stimuli and the responses of an individual via regulating energy allocation and numerous other physiological processes as well as behavior and are therefore of special interest in ecophysiological studies. The immune and the oxygen delivery system represent two vital body functions that are essential for survival and respond sensitively to altered environmental conditions, nutrient deficiencies and stress hormone levels, representing therefore further suitable targets of investigation in ecophysiological studies.

Our study species, the edible dormouse (*Glis glis*) is a small arboreal rodent (~ 100 g) characterized by an extraordinarily long hibernation period of up to eight months and a highly synchronized yearly cycle of animals within a population. The aim of this study was to elucidate physiological mechanisms underlying the evolution of life history strategies and variations in fitness parameters associated with stressful and demanding situations like hibernation, reproduction, limited food availability and high population density. To achieve these goals urinary GC levels as well as white and red blood cell (WBC and RBC) parameters were investigated and urine samples were examined for haemoglobinuria of free ranging edible dormice of five different study sites in South Western Germany.

Results of this study reveal that the post-hibernation period represents an extremely challenging period for edible dormice as their phagocytic cells, the immunological first line of defense, obviously become depleted during the extended hibernation period and recover only slowly at the beginning of the active season. The need to invest into the restoration of regressed organs and bodily functions directly after hibernation when high quality food is still limited, may explain the delayed recovery of these innate immune cells. Slightly elevated cortisol levels presumably reflect the mobilization of energy from body stores for these restoration processes. As the phenomenon of low phagocyte counts was even more pronounced at the beginning of a low food year and the few neutrophils present in the blood of dormice were primarily immature, preparatory mechanisms occurring during late arousals of the hibernation period seem to determine the regeneration of phagocytes before hibernation is terminated. This, in turn, indicates that dormice are able to predict upcoming food availability and, consequently, future reproductive effort and accordingly invest into physical-

physiological recovery. Apparently edible dormice trade off restoration of regressed organs for immunity. Survival probabilities of edible dormice are lowest at that time of the annual cycle, suggesting that this post-hibernation impairment of the innate immune system may entail detrimental effects for their fitness.

Elevated cortisol levels during mating and gestation-lactation, respectively, show that reproduction represents a stressful life history event in both sexes. This event furthermore coincides with drastic increases in the ratio of phagocyte to lymphocyte counts (P/L ratio), a stress response of the immune system, as well as distinct impairments in the oxygen delivery system. The latter seems to be in a large part due to energetic and nutrient deficits as well as simultaneously occurring large amounts of senescent RBCs. High frequency of haemoglobinuria especially in females support the notion of a nutrient-deficient anaemiaWhen all reproductive effort has ended, cortisol levels decrease whereas the strong increases in the P/L ratio persist until the end of the active season which gives notice of the prolonged immunological effects of chronic stress. As mortality is increased during reproductive years, the high cortisol levels measured reproductive activity suggest an allostatic overload that has exceeded adaptive levels and the high P/L ratios might represent a pivotal indicator for the beginning of a stress-induced deprivation of immune function that contribute to reduced survival.

Prolonged food limitation does not elevate cortisol levels and P/L ratios, supporting the hypothesis that food availability is predictable for edible dormice and that a restriction therefore does not cause considerable stress. During late summer of a low food year the oxygen delivery capacity is decreased, as revealed by lower RBC counts and haemoglobin concentration which is usually interpreted as an impairment in body condition. However, erythrocyte parameters furthermore indicate a senescent RBC pool, suggesting that a reduced erythrocyte production is part of the energy saving strategy. As survival is increased during years of low food availability, these findings indicate that dormice are able to perfectly adapt to prolonged periods of limited food availability.

Summarizing, urinary cortisol concentrations in edible dormice precisely reflect adjusted hormonal set points to different situations and thus provide information on variations in allostatic load. RBC and WBC parameters appear to be reliable indicators for the evaluation of the physiological effects of challenging conditions and have the potential to reveal physiological and fitness trade-offs. The results of this study highlight that in-depth investigations of cortisol levels and haematological parameters over an extended period are critical for disentangling the impact of different stressors and obtaining a comprehensive understanding of these complex relationships.

Chapter I: General Introduction

In a complex interplay of the biochemical, neuronal and endocrine networks organisms steadily respond to changed environmental as well as internal conditions. Animals living in the wild are generally confronted with limited resources, hence, the allocation of resources into different physiological processes typically result in trade-offs between competing body functions, especially during demanding situations (Harshman and Zera, 2007; Speakman, 2008). Such trade-offs may have negative consequences on the performance and fitness of the individual, such as a reduced current or future reproductive outcome or increased mortality (e.g. Harrison et al., 2011; Sanderson et al., 2014). The investigation of individual physiological responses to environmental conditions during different life history stages may therefore be the key to understand mechanisms underlying demographic changes and species declines. The field of conservation physiology hence connects physiological responses upon environmental challenges with the two fitness components survival and reproductive success. Suitable targets of investigation are body functions that are vital for the whole organism and known to respond sensitively to stressors or changed environmental conditions, like e. g. endocrine regulation, metabolism, immunity and the oxygen delivery system (e.g. Lochmiller and Deerenberg, 2000; Ricklefs and Wikelski, 2002; Wikelski and Cooke, 2006). Endocrine mediators, especially the glucocorticoid hormones (GCs) that constitute the end product of the hypothalamic-pituitary-adrenal (HPA) stress axis, represent a central link between environmental changes and physiological, behavioural as well as morphological responses of individuals (Glaser and Kiecolt-Glaser, 2005; Sapolsky et al., 2000). Changes in the GC level reflect functional shifts with the aim to adjust to changed environmental conditions or requirements of certain life history traits, as highlighted by the concept of allostasis, which describes the process of "achieving stability through change" (McEwen and Wingfield, 2003, 2010). Accordingly, elevations in GC enables the individual to cope with strenuous situations, like e.g. reproduction, however, extremely or chronically increased GC levels may entail negative consequences for the health and the performance of an individual (Sapolsky et al., 2000; Webster Marketon and Glaser, 2008). The disentanglement of fitness-enhancing from fitness-depriving stress hormone effects and the identification of the underlying effectors is a challenging task, especially under natural conditions. Therefore, the evaluation of the responses of additional physiological systems that are vital for the organism and affected by changes in GC levels is recommendable. The detailed investigation of life conditions and associated changes in GC levels as well as further physiological measures helps to assess the impact of different stressors on different physiological functions and elucidate mechanisms underlying variations in fitness and causes of population declines.

Under stressful situations, a quick first surge of catecholamines is released via the sympathetic nervous system into the circulating blood, whereas the HPA axis that culminates in the release of GCs reacts time-lagged and maintains the stress response for a longer period of time. GCs are therefore the ideal candidate to detect the occurrence of persistent stress (Goldstein, 2003; Lundberg, 2005; Sapolsky et al., 2000). One major function of GCs is the provisioning of energy by increasing glucose levels in the blood, furthermore, they act on a multifold of cell types and thereby influence numerous body functions and the resource allocation among them (Glaser and Kiecolt-Glaser, 2005; Sapolsky et al., 2000; von Holst, 1998), including the immune and the oxygen delivery system. Immune and red blood cells (RBCs) are indispensable for survival and underlie remarkable variations in response to changing and demanding conditions. Both furthermore require a substantial amount of energy and micronutrients and consequently compete for resources with other body functions, above all, growth, development, and reproduction, eventually resulting in trade-offs between these fitness-relevant physiological functions (Buttgereit et al., 2000; Cutrera et al., 2010; Keohane et al., 2015; King and Swanson, 2013; Lee, 2006; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). Accordingly, food availability and quality can distinctly impact the numbers of white blood cell (WBC) subtypes, immune functionality and erythrocyte generation (Brock et al., 2013; Ritz and Gardner, 2006; Tyler and Cowell, 1996).

Chronic stress is associated with detrimental effects on health, including cardiovascular diseases and impairments in immunity, such as reduced immune functions, increased susceptibility to infections, slow wound healing, and recurrent virus infections (Glaser and Kiecolt-Glaser, 2005). In wild animals, chronic stress has been connected to reduced breeding success and survival (Elenkov et al., 1999; Ellenberg et al., 2007; Wikelski and Cooke, 2006). Constantly increased GCs elevate the phagocyte:lymphocyte (P/L) ratio, i.e. phagocytic immune cells (neutrophils and monocytes) augment whereas lymphocytes in the circulation decrease. These alterations are conveyed by increasing the haematopoietic mediators granulocyte- and granulocyte-macrophage colony-stimulating factor (G-CSF and GM-CSF), that boost the generation of phagocytes in the bone marrow, enhance survival and reduce vessel wall adherence of phagocytes (Cebon et al., 1994; Cronstein et al., 1992; von Vietinghoff and Ley, 2008; Weyts et al., 1998). At the same time, GCs induce lymphocyte extravasation and apoptosis (Penninger and Mak, 1994; Wang et al., 2002). Thus, increases in the P/L ratio represent a stress response on the immune system level. The relevance of this increase for health has been discussed controversially. On the one hand, immunoenhancing effects were hypothesised, proposing that leukocytes might be redistributed to the sites of pathogen encounter (Dhabhar et al., 1995). On the other hand, enhanced lymphocyte apoptosis and the fact that the reduced neutrophil extravasation hampers their immunologic action by inhibiting neutrophil recruitment to sites of pathogen encounter (Anderson and Springer, 1987) emphasize the negative

effects underlying these alterations in immune cell counts. This is furthermore supported by the effective application of GCs against autoimmune reactions and the well-known detrimental implications of chronic stress for health (Dhabhar, 2002; Glaser and Kiecolt-Glaser, 2005).

RBCs are of special interest due to their important vital function of delivering oxygen to body tissues and their susceptibility to impairments in cell generation. A reduction in RBC counts and haemoglobin can result in anaemia which is associated with severe implications on health and well-being, such as fatigue, dyspnea, cardiovascular and neurological implications (Lipschitz, 2003; Tyler and Cowell, 1996). A multitude of diseases and deficiencies may cause an anaemic state. For example, erythropoiesis is hampered by irradiation, malnutrition and fasting as well as disturbances of hormones involved in haematopoiesis (e.g. erythropoetin; Hoffman et al., 2013; Koury and Ponka, 2004; Tyler and Cowell, 1996), whereas RBC lifespan may be curtailed by energy depletion, chemical intoxication, mechanical rupture due to shear forces or osmotic and oxidative stress, the latter being the most common form of accelerated RBC aging and destruction (Lang et al., 2005; Lutz and Bogdanova, 2013; Sivilotti, 2004). Stress hormones are not the main driver of anaemia, but GCs can enhance premature erythrocyte death by exacerbating oxidative stress and compromising protective antioxidant systems (Fibach and Rachmilewitz, 2008; Oishi et al., 1999; Orzechowski et al., 2002). At the same time, GCs enhance RBC production and efflux from the bone marrow (Fisher and Crook, 1962; Lodish et al., 2010), that may support a fast recovery during anaemia. In general, a state of considerably increased erythrocyte destruction, caused e.g. through haemorrhage or haemolytic disorders, results in a regenerative reaction of the bone marrow, namely an accelerated RBC generation to restore the oxidative capacity. This is usually hallmarked by an intensified liberation of the immature reticulocytes (Aslinia et al., 2006), that are larger and less capable to load oxygen than mature RBCs. A comprehensive set of haematological indices helps to characterize and to identify the potential causes underlying impairments in the oxygen delivery system. An additional and useful indicator for assessing the oxygen transport system can be the urine colour as extreme haemolytic processes result in haemoglobinuria, which becomes obvious in urine colored reddish-brown to brown-black (Keohane et al., 2015).

Fasting and malnutrition, reproductive effort, pathogen load and habitat quality have been shown to affect leukocyte counts and the oxygen transport system, sometimes resulting in distinct characteristics (Brock et al., 2013; Ito et al., 1964; Jégo et al., 2014; Johnstone et al., 2012; Lochmiller et al., 1993; Tyler and Cowell, 1996). For these reasons, an in depth and comprehensive blood picture, encompassing a white blood cell differential and a broad set of erythrocyte indices, is a good indicator for demanding life history events and environmental challenges and gives notice of health impairments. Many studies on wildlife so far concentrate only on the impact of a single external factor on these parameters or examine few parameters such as total WBC counts or RBC counts

and/or haematocrit (Gilot-Fromont et al., 2012; Ots et al., 1998). As organisms are faced with a variety of challenges during their yearly cycle, the measurement of GC-levels is useful to determine the allostatic load associated with different environmental conditions and life history states, while an in-depth examination of the WBC and the RBC picture gives notice of their impact on health and performance. This complete picture may help to disentangle different effectors and to elucidate mechanisms underlying variations in health and causes of population declines.

Our study species, the edible dormouse (*Glis glis*) is a small arboreal rodent (~ 100 g) characterized by an extraordinarily long hibernation period of up to eight months between the end of September until the end of May (Schlund, 2005). Hibernation represents an extreme and the most efficient physiological adaptation to reduce energy expenditure during unfavorable environmental conditions. During extended single torpor bouts (Bieber et al., 2014) heart and metabolic rates are drastically diminished, accompanied by a strongly reduced body temperature down to ambient values (Barnes, 1989; Bieber et al., 2014; Geiser et al., 1990; Ruf and Geiser, 2015; Wilz and Heldmaier, 2000). Dormice are fat storing hibernators, which means that they store body fat prior to and completely cease feeding during hibernation (Fietz et al. 2005). In Central Europe, this nocturnal rodent occurs preferentially in deciduous mixed forests dominated by European beech (*Fagus sylvatica*; Schlund 2005). It mates in July and produces one litter per year. After a gestation period of about 30 days, litters averaging 5-6 young are born in August (Fietz et al., 2009). For a small mammal, edible dormice are comparatively long-lived and may reach ages of up to 12 years with an average longevity of 3-4 years in nature (Ruf et al., 2006). This species represents for several reasons an excellent study organism to investigate causes of individual and seasonal variability of physiological parameters and their consequences for fitness. Dormice frequently use nest boxes to rest during the day and to rear their offspring, which makes them easily accessible for scientific studies. Its whole life history is closely adapted to the irregular seed production of their main feeding tree species, the European beech (Schlund, 2005), resulting in high reproductive activity in full mast years and whole populations that skip reproduction in years of mast failure (Lebl et al., 2011; Ruf et al., 2006). Caused by irregular seed production this small rodent is confronted with extended periods of food scarcity that may last longer than 1.5 years. As individuals within one population are highly synchronized in their yearly cycle this system provides natural experimental conditions in which trade-offs should become apparent even on a population level. Whereas most ecoimmunological studies cannot link immunological data to survival rates as a major component of fitness, it is known from former studies that high survival rates in edible dormice occur during years of food limitation and survival probabilities in both sexes are reduced after years of high food availability and high reproductive investment (Lebl et al., 2011; Ruf et al., 2006). However, mechanisms underlying reduced survival

rates were up to now unknown. Moreover, population densities of edible dormice show strong local variations, which offers the unique opportunity to investigate if and to what extend high population densities contribute to perceived physiological stress and health impairments.

The aim of this study was to elucidate physiological mechanisms underlying the evolution of life history strategies and variations in fitness parameters associated with stressful and demanding situations like hibernation, reproduction, limited food availability and high population density. To achieve these goals we investigated urinary GC levels as well as WBC and RBC parameters in the blood and examined urine samples for haemoglobinuria of free ranging edible dormice of five different study sites in South Western Germany.

With regards to the detailed knowledge of the ecology of this species, this approach allows to identify challenging conditions and characterize associated allocation decisions (Lee, 2006; Sheldon and Verhulst, 1996). Thereby, physiological interrelations and functional trade-offs that may underlie declines in individual performance and population density may be elucidated in the wild.

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Chapter II: The leukocyte differential

It takes two to tango: phagocyte and lymphocyte numbers in a small mammalian hibernator

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Abstract

Immunity is energetically costly and competes for resources with other physiological body functions, which may result in trade-offs that impair fitness during demanding situations. Endocrine mediators, particularly stress hormones, play a central role in these relationships and directly impact leukocyte differentials. To determine the effects of external stressors, energetic restraints and competing physiological functions on immune parameters and their relevance for fitness, we investigated leukocyte profiles during the active season of a small obligate hibernator, the edible dormouse (*Glis glis*), in five different study sites in south-western Germany. The highly synchronized yearly cycle of this species and the close adaptation of its life history to the irregular abundance of food resources provide a natural experiment to elucidate mechanisms underlying variations in fitness parameters. In contrast to previous studies on hibernators, that showed an immediate recovery of all leukocyte subtypes upon emergence, our study revealed that hibernation results in depleted phagocyte (neutrophils and monocytes) stores that recovered only slowly. As the phenomenon of low phagocyte counts was even more pronounced at the beginning of a low food year and primarily immature neutrophils were present in the blood upon emergence, preparatory mechanisms seem to

determine the regeneration of phagocytes before hibernation is terminated. Surprisingly, the recovery of phagocytes thereafter took several weeks, presumably due to energetic restrictions. This impaired first line of defense coincides with lowest survival probabilities during the annual cycle of our study species. Reduced survival could furthermore be linked to drastic increases in the P/L ratio (phagocytes/lymphocytes), an indicator of physiological stress, during reproduction. On the other hand, moderate augmentations in the P/L ratio occurred during periods of low food availability and were associated with increased survival, but reproductive failure. In this case, the stress response probably represents an adaptive reaction that contributes to survival by activating energy resources. In contrast to our expectation, we could not detect an amplification of stress through high population densities. Summarized, results of our study clearly reveal that the leukocyte picture of active edible dormice responds sensitively to physiological conditions associated with hibernation, reproductive activity and food availability and can be linked to fitness parameters such as survival. Thus edible dormice represent an excellent model organism to investigate regulatory mechanisms of the immune system under natural conditions.

1. Introduction

In vertebrates immune defense is essential for survival and varies remarkably among individuals and in time. The interaction of the immune system with different physiological processes, the costs of each of them as well as a variety of external stressors represent potential causes for this strong variability (Lochmiller and Deerenberg, 2000; Martin, 2009). The pure maintenance of the immune system already consumes considerable amounts of energy (Buttgereit et al., 2000; Straub, 2012), with further substantial increases upon mounting an immune response (Clark et al., 1996; Cooper et al., 1994; Cutrera et al., 2010; King and Swanson, 2013; Ksiazek et al., 2003; Roe and Kinney, 1965). In line with these observations, nutritional status and food quality were shown to affect the numbers of certain leukocyte subtypes in the blood and distinct immune functions (Brock et al., 2013; Lochmiller et al., 1993; Ritz and Gardner, 2006; Saino et al., 1997). If confronted with limited resources animals in the wild counterbalance different physiological demands at the expense of their immune defense (Hanssen et al., 2004; Lee, 2006; Lochmiller and Deerenberg, 2000; Martin et al., 2008; Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996). Thus, being energetically costly, immune defense inevitably competes for resources with other body functions, above all growth, development, and reproduction, leading to a trade-off between these fitness relevant physiological processes (Ardia et al., 2003; Hanssen et al., 2004; Lee, 2006; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Speakman, 2008; Zuk and Stoehr, 2002).

Endocrine mediators play an important role in the regulation of immunity. Under stressful situations, stress hormones (catecholamines and glucocorticoids, GCs) are released into the blood stream, acting on multifold tissues and genes and thereby influencing e.g. growth, metabolism and resource allocation (Bartolomucci, 2007; Glaser and Kiecolt-Glaser, 2005a; von Holst, 1998; Khansari et al., 1990; Noble, 2002; Sapolsky et al., 2000). If stressors persist for an extended period of time or reappear frequently, we refer to chronic stress, which is associated with detrimental effects on immune system and health, including an increased susceptibility to infections, impaired immune responses, slow wound healing and recurring virus infections (Altemus et al., 2001; Dhabhar et al., 1996; Glaser and Kiecolt-Glaser, 2005; Millán et al., 1996; Sapolsky et al., 2000; Silberman et al., 2003; Tsigos and Chrousos, 2002). In wild animals, stress may directly compromise fitness, as chronically stressed individuals were shown to suffer from reduced breeding success and survival (Ellenberg et al., 2007; Müllner et al., 2004; Wikelski et al., 2002). Concerning the differential blood count, augmented GC levels result in increased numbers of phagocytes (P), namely neutrophils (N) and monocytes. GCs elevate their principal haematopoietic mediators G-CSF/GM-CSF and thereby boost their release from the bone marrow, while simultaneously reducing apoptosis and vessel wall adherence (Cebon et al., 1994; Cronstein et al., 1992; Jilma et al., 1998; Kawakami et al., 1990;

Mishler and Emerson, 1977; Sapolsky et al., 2000; von Vietinghoff and Ley, 2008; F. A. Weyts et al., 1998). On the other hand, they reduce lymphocyte (L) counts in peripheral blood through extravasation as well as enhanced apoptosis (Dhabhar et al., 1996, 1994; Penninger and Mak, 1994; Wang et al., 2002; F. A. A. Weyts et al., 1998). The stress-induced L apoptosis is mediated by "death signals" exerted from stressed innate immune cells (Kono et al., 2001) and the accumulation of the death receptor Fas on L, but requires prolonged stress signals in comparison to L extravasation (Nagata and Golstein, 1995; Penninger and Mak, 1994; Shi et al., 2003). Thus, under chronic stress, circulating cell numbers of the adaptive immune system are down-regulated, while the innate immune cell numbers that form the first line of defense in any infection are up-regulated. Studies reporting on the connection of elevated N/L ratios (neutrophils/lymphocytes) with a high cardiovascular risk and susceptibility to infections in humans, slow growth rates and low survival in wild animals, confer the predictive power to this measure (Davis et al., 2008). Equivalent to the N/L ratio, a high P/L ratio is a measure of physiological stress with functional significance, as chronic stress elevates both monocytes and N (Heidt et al., 2014; Oishi et al., 1999)

Animals have different options to adapt to energetically challenging situations, like extended periods of food limitation. The physiologically most efficient way to reduce energy consumption and to survive periods of unfavorable environmental conditions is to enter hibernation. During hibernation the organism undergoes extreme physiological changes, like drastically diminished heart and metabolic rates (Geiser and Ruf, 1995), accompanied by the reduction in body temperature (T_b) down to ambient temperature, in extreme cases down to 0°C or even below (Barnes, 1989; Geiser et al., 1990), as well as the regression of organs, like e.g. the gonads and the intestine (Carey et al., 2003; Hume et al., 2002). These severe physiological changes also concern various components of the immune system. Accordingly, during torpor a dramatic decrease (~ 90%) in circulating leukocytes occurs, that applies to all subtypes, but cell numbers are restored within few hours after termination of the torpor bout (*Cricetus cricetus:* Reznik et al. 1975; *Erinaceus europeaus L*.: Suomalainen and Rosokivi 1973; *Ictidomys tridecemlineatus:* Spurrier and Dawe 1973; *Spermophilus citellus*: Bouma et al., 2010; *Urocitellus parryii*: Toien et al. 2001). Typically, hypothermic phases are interspersed by arousals, during which T_b is shortly elevated to euthermic values (Geiser and Ruf, 1995). The function of these energetically extremely costly arousals remains elusive. Several investigations give rise to the assumption that they are necessary for the maintenance of vital body functions throughout the long hibernation period, including immune defense and the development and maturation of tissues and organs (Kruman et al., 1988b; Kruman, 1992; Prendergast et al., 2002).

Our study species, the edible dormouse (*Glis glis*) is a small arboreal rodent characterized by an extraordinarily long hibernation period of up to 8 months that consists of extended single torpor bouts (Bieber et al., 2014) and represents an excellent study organism to investigate causes of

individual and seasonal variability in immune parameters and its consequences for fitness. Its whole life history is closely adapted to the irregular seed production of their main feeding tree species, the European beech (*Fagus sylvatica*), resulting in high reproductive activity in full mast years and whole populations that skip reproduction in years of mast failure (Lebl et al., 2011; Ruf et al., 2006). Caused by irregular seed production this small rodent is confronted with extended periods of food scarcity that may last longer than 1.5 years. As individuals within one population are highly synchronized in their yearly cycle, this system provides natural experimental conditions in which trade-offs should become apparent even on a population level. Whereas most ecoimmunological studies cannot link immunological data to survival rates, one main component of fitness, we know from former studies that high survival rates occur during years of food limitation, whereas survival rates of both sexes were reduced during years of high food availability due to high reproductive investment (Lebl et al., 2011; Ruf et al., 2006). Moreover, population densities of edible dormice show strong local variations, which offers the unique opportunity to investigate if and to what extend high population densities contribute to perceived physiological stress and impair immunocompetence under natural conditions.

The aim of this field study was to elucidate physiological mechanisms underlying variations of fitness parameters associated with stressful and demanding situations like hibernation, reproductive activity, variations in food availability and population density. We therefore analyzed blood samples of free ranging adult male and female edible dormice of five different study sites in South-Western Germany. We predicted that the prolonged hibernation period associated with extreme physiological conditions has a considerable impact on the leukocyte differential, including a clearly detectable recovery phase after hibernation is terminated. As reproduction might represent stress for both sexes we expected to detect stress-specific alterations in the WBC differential associated with high reproductive investment, such as an elevation in the P/L ratio. Since maintenance of the immune system is associated with substantial energetic costs, another important aim of our study was to investigate the effect of prolonged food limitation on WBC production. We expected lower WBC counts during extended periods of food limitation in comparison to the time of seed masting. However, besides energetic restrictions, prolonged fasting periods might also provoke chronic stress and may furthermore elevate the P/L ratio. Thus we expected a combination of low WBC counts with high P/L ratios through fasting. High population densities might amplify stressful situations associated with intraspecific competition for resources and mating opportunities. We therefore expected stress-induced effects on immune parameters to become pronounced in high density populations.

2. Material and Methods

2.1. Study animal

The arboreal edible dormouse (*G. glis*) is the largest European dormouse with a body mass of around 100 g (Schlund, 2005). However, body mass reveals strong seasonal variations and even though sexes do not differ in their body mass at the onset of hibernation, males emerge from hibernation slightly heavier than females but drop below females' body masses during the mating as well as gestation/early lactation season (Fietz et al. unpubl. data). In Central Europe, this nocturnal rodent occurs preferentially in deciduous mixed forests dominated by European beech (*Fagus sylvatica*; Schlund 2005). In Germany, this obligate hibernator spends approximately 8 months hibernating in underground burrows, generally from the end of September until the end of May (Vietinghoff-Riesch, 1960). During hibernation, body temperature is drastically reduced close to ambient temperature (Bieber et al., 2014) and metabolic rate to a fraction of the euthermic values (Wilz and Heldmaier, 2000). As a fat storing hibernator, dormice cease feeding and rely entirely on stored fat accumulated during the previous autumn for energy metabolism (Fietz et al., 2005). Typical for hibernators, dormouse males regress their testes before hibernation starts and develop them in size and function after emergence. Males emerge from hibernation 2-3 weeks earlier than females (Schlund 2005). In Germany, dormice produce maximum one litter per year and mating takes place in July. After a gestation period of about 30 days, litters averaging 5-6 young are born in August (Fietz et al., 2009; Vietinghoff-Riesch, 1960). Edible dormice are typical slow-living species as they have low reproductive rates and may reach ages of up to 11 years with an average longevity of 3-4 years in nature (Lee, 2006; Ruf et al., 2006). Dormice frequently use nest boxes to rest during the day and to rear their offspring, which makes them easily accessible for scientific studies.

2.2. Study sites

This study was conducted at five different sites located in mixed deciduous forests in south western Germany (Tab. 1). The first study site (HE) is located near the town Tübingen at the southern rim of the “Schönbuch” Nature Reserve, which represents one of the largest continuous woodlands within the state of Baden-Württemberg. The four other study sites were located within forest fragments of varying sizes (11-135 ha) close to the town Ulm, about 70 km south east of Tübingen (for details on study sites and edible dormouse populations see Tab. 1 in Fietz et al., 2014). Dormouse populations of all study sites differed distinctively in their density (Tab. 1). In each study site, nest boxes were installed three meters above ground at the intersections of grid lines marking 30×30 m squares.

Table 1. Locations including coordinates, sizes of forests and study sites, number of individuals (except juveniles) captured during the whole study period (2012-2014).

Study site	Locations near the city	Coordinates	Forest size [ha]	Size of study site [ha]	Number of nest boxes	Total number of individuals captured in 2012 - 2014 (individuals/ha)
HE	Tübingen	48°33'03.38''N 8°59'59.72''E	15.000	20.4	228	14.1
KW	Ulm	48°22'56.50''N 9°55'54.45''E	135	7	70	18.7
BG	Ulm	48°25'22.36''N 9°57'43.17''E	70	7	70	64.9
BS	Ulm	48°29'21.18''N 9°58'219.02''E	33	7	70	41.1
JH	Ulm	48°22'11.13''N 9°49'3.44''E	11	7	70	26.6

2.3. Capture-mark-recapture & Measurements

We checked the nest boxes at all study sites during daytime at bi-weekly intervals from the end of May through September in 2012 to 2014. Upon first capture, we marked each individual with a transponder (Trovan, EURO I.D. Usling, Weilerswist, Germany). We recorded sex, body mass using a 300 g spring balance (Pesola, Baar, Switzerland; division: 2 g, accuracy: 99.7%) and tibia length and in males testes length to the nearest 0.1 mm using a sliding caliper. Tibia length was used as a proxy for body size. Body condition is defined as body mass (in g) divided by tibia length (in mm). Reproductive activity was determined in males by checking for the presence of tangible testis. As dormouse males remain sexually quiescent during years of mast failure, testes are only visible and tangible shortly before and after the mating season in masting years (Fietz et al., 2004). Females were classified as reproductive when showing clear signs of gestation (detected by the palpation of the abdomen) or lactation.

2.4. Blood sampling

To avoid potential effects in white blood cell differential counts caused by acute stress during handling, blood sampling was performed as fast as possible after first disturbance of the study animal. In more than 90 % of all cases we sampled the blood within 3:30 min, only in 6 cases it took longer than 12 min. However, we statistically excluded an effect of sampling duration on leukocyte profiles (see below 2.6). Approximately 50 µl blood was collected into EDTA-coated tubes (Sarstedt, Microvette 200) by punctuation of the *V. fascialis* with a hollow / 23-gauge needle. Samples were

subsequently stored for a maximum of 8 hours in a cool bag until processing in the lab. Blood smears were prepared directly in the field. During 2012 we exclusively sampled blood of adult males, whereas in 2013 and 2014 blood samples were collected from individuals of both sexes that were one year old at least. 143 of 248 individuals were sampled twice, most of them within 14 days.

2.5. WBC parameters

In 2012, smears of all blood samples were Pappenheim-stained for obtaining WBC differentials and for a subset of samples WBC counts were determined with a Coulter Counter (Beckman Coulter, Brea, USA) by diluting 5 µL of whole blood in 10 mL of buffer and adding two drops of ZAP-Oglobin II Lytic Reagent (Beckman Coulter, Brea, USA). In 2013 and 2014, a haematology system (pocH-100i, Sysmex, Japan) was used to obtain WBC counts (WBC/µl) and proportions of L and P, spending 12 µl EDTA blood. Coulter Counter measurements differed on average by 1.21±0.96% (n=14) from cell numbers measured with the pocH-100i. In a further approach, a subset of blood samples of 2013 and 2014 was analyzed by Pappenheim-stained blood smears. Blood smears were assessed to confirm the results of the haematology system and for obtaining additional and more detailed information on the leukocyte differential, such as on different developmental stages of the neutrophils, which were of special interest for this study. For this analysis at least 400 cells were counted per blood sample. By blood smear evaluation, leukocytes were differentiated into lymphocytes, monocytes, neutrophils, basophils and eosinophils, whereas neutrophils were discriminated into the mature segmented neutrophils and the immature band neutrophils and metamyelocytes. Monocytes represent a negligible proportion (<2%) of the leukocytes. We used the P/L ratio which is the number of P (P=monocytes+neutrophils) divided by the number of L to measure stress-induced physiological changes, equivalent to the N/L ratio used in former studies (Davis et al., 2008), as monocytes constitute like neutrophils phagocytic cells of the innate immune system that are elevated in numbers by chronic stress (Heidt et al., 2014; Oishi et al., 1999).

Based on the biology and physiology of the edible dormouse, and their strongly synchronized yearly cycle, we defined three distinct time periods during the active season of this species: the post-hibernation period (PostH), the reproductive period (Rep) and the pre-hibernation period (PreH). The post-hibernation period starts directly after hibernation is terminated and lasts until the onset of the reproductive period. In case of WBC parameters that showed a clear dynamic at the beginning of the active period, we subdivided this first period further into two separate time periods to specifically assess hibernation effects: a short post-emergence period (PE; 3 weeks after the first capture), which represents the physiological status upon emergence and a recovery period (R) which covers the remaining time before the onset of reproductive activities. The timing of the main reproductive

investment differs among sexes, in males the reproductive period is represented by the mating season, whereas in females this period refers to the period of late gestation and lactation. The pre-hibernation period, finally, serves for the accumulation of fat reserves and partly coincides with the raising of young by females.

2.6. Statistical analyses

2013 and 2014 were years of high beech seed production and high dormouse reproductive activity in all study sites. 2012 was a year of mast and reproductive failure except for study site BS, where 2012 represented also a high food and reproductive year. To avoid a biased data set, BS was excluded for the comparative analyses concerning the effect of reproductive activity and food availability between low and high food and reproductive years. For the effect of food availability on WBC parameters we focused on males, because in females the availability of high quality food coincides with high reproductive investment and it is therefore impossible to disentangle these two effects in females.

We used R 3.0.3 (R Development Core Team, 2012) and the package *lmerTest* (Kuznetsova et al., 2014) to perform linear mixed effects analyses of the relationships between haematological parameters and the different fixed effect variables “period” (four levels: P, R, Rep, PreH), “Julian day”, “population density” (five levels according to the different study sites), “body condition” and “food/repro”. Note that in edible dormice reproduction is synchronized with the masting pattern of the beech. The factor "food/repro" can adopt two levels: high versus low. High means that food availability is high and virtually all dormice reproduced within this year (2013 and 2014), low on the other side means, that beeches and dormice completely failed to reproduce during the respective year (2012). To get p-values from the mixed effects models we used the package *lmerTest* (Kuznetsova et al., 2014), which uses the Satterthwaite approximation to determine degrees of freedom. We corrected for potential effects of “study sites” (five levels), time of day, sampling duration (in seconds) and recapture, by including them as independent variables into the models. An effect of recapture or sampling duration was never detected. We further included “individual” as random effect. Data were transformed if necessary to achieve normality and homoscedasticity in the mixed model (MM) and were assured by visual inspection of histograms and plots of fitted values against residuals. The functions *summary* and *Anova (type 3)* were used to obtain results of the models. Test results were considered significant, if p was <0.05. All non-significant independent variables were excluded stepwise from the final model. Details of the particular models used are given within the respective results section. In Figures, asterisks are used to display significance levels according to MMs with the following indications: “**.**” p<0.1; “*” p<0.05; “**” p<0.01; ” ***” p<0.001

3. Results

3.1 Blood samples collected

In total we collected blood samples of 248 edible dormice between May and September in 2012 until 2014 in five different study sites. 40 adult males were sampled in 2012, a year of reproductive failure in edible dormice (except in the study site BS) and low beech seed masting. During 2013 and 2014, years of high beech seed production and high dormouse reproductive activity, 103 females and 105 males were sampled. Sample sizes are evenly distributed among study sites.

3.2. Seasonal variations in WBC parameters during the high food/reproductive years 2013 and 2014

In males P counts were lowest upon emergence from hibernation (2,800±850 P/µL; n=31), increased constantly until the end of the mating season and remained on a comparably high level towards the end of their active period (Table A.1; Fig. 1). In contrast to the P counts, L counts were high (10,010±3,330 L/µL; n=31) after emergence and remained on a constant level until the start of the mating season. During the mating season L counts dropped sharply by about 25% and continued to decrease during the subsequent pre-hibernation period (Table A.2; Fig. 1). As a consequence of increasing P and decreasing L numbers, the P/L ratio in males showed a distinct and sharp increase during the mating season and remained on a constant and high level thereafter (Table A.3; Fig. 1). The blood smear evaluation revealed that also the number of monocytes varied significantly between the different periods of the active season (ANOVA, p=0.018). Accordingly, monocyte numbers increased during reproduction compared to the post-emergence period in males (Model: sqrt monocyte = period+ID random; n=67; $estimate_{Rep}$=0.106; p_{Rep}=0.061).

In females P counts accounted for 2,991±845 P/µL (n=17) when hibernation was terminated and in contrast to the males, these numbers increased significantly throughout their entire active season (Table A.4; Fig. 1). Female L numbers (9,329±1,445 L/µL; n=17) were lower compared to that of the males at the beginning of their active period (Model: lgL = julian day+sex+study site+ID random; n= 60; $estimate_{males}$= 1.236; p_{males}=0.079) and increased slightly thereafter until the start of their reproductive period. During the period of high female reproductive investment (late gestation and lactation) until the end of their active season, L counts showed a drastic decline (Table A.5; Fig. 1). Seasonal changes in P and L counts resulted in a constantly augmenting P/L ratio in females throughout their entire active period (Table A.6; Fig. 1).

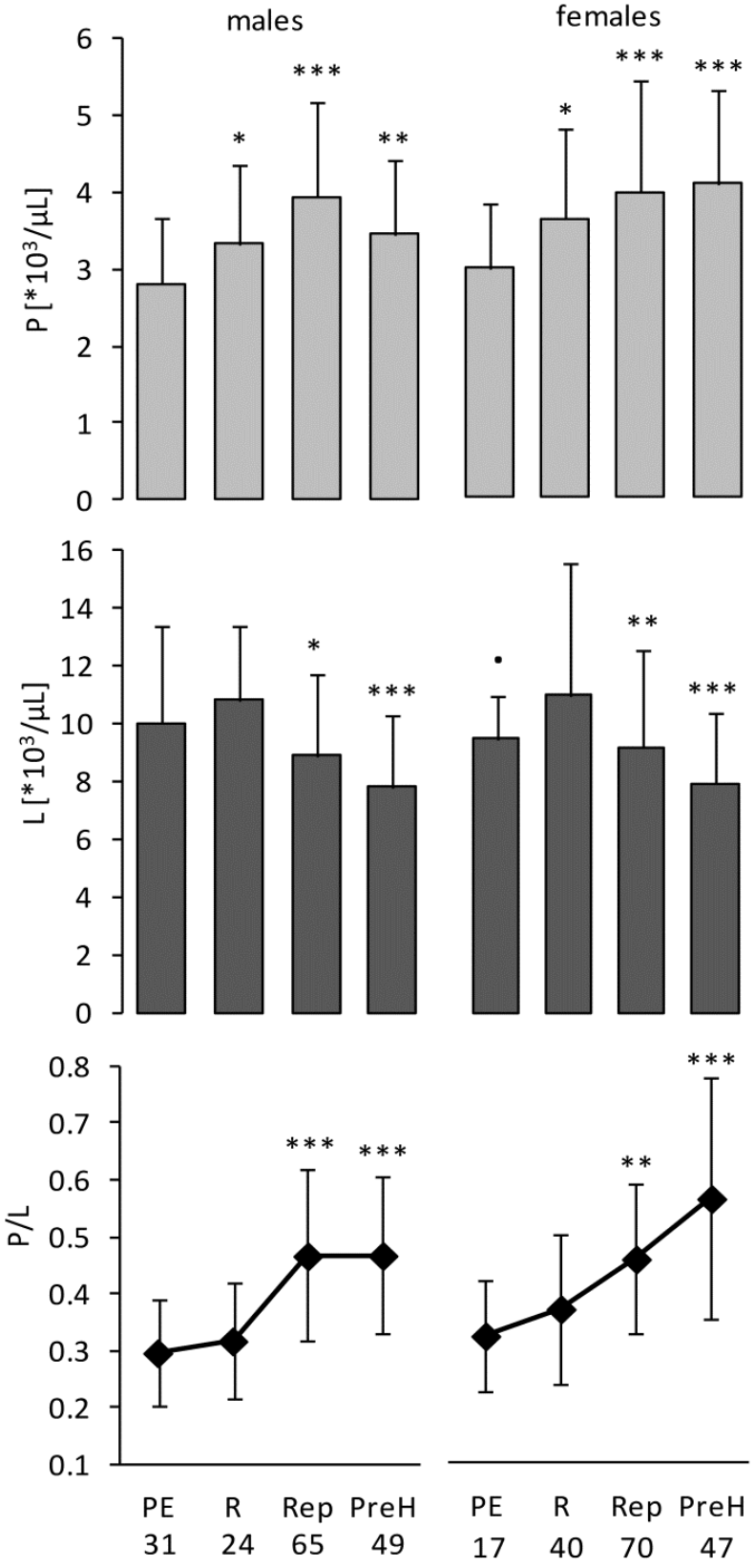

Fig. 1. Seasonal changes of P counts (top), L counts (middle) and P/L ratios (below) in male (left) and female (right) edible dormice during their active period in the high food/repro years 2013 and 2014 (mean and SD). Significant variations among periods were determined in comparison to PE as a reference for P counts and P/L ratios and in comparison to R as a reference for L counts.

The additional evaluation of a subset of blood smears for the assessment of the developmental stages of immune cells revealed that Ns constituted the largest proportion of the phagocytic cells, whereas monocytes ranged on very low levels, accounting only for 0.66% (SD=0.9; n=26) of total WBC counts in females and 1.18% (SD=1.55; n=78) in males. During the entire active season the proportion of band neutrophils was extremely high and metamyelocytes were regularly present, together accounting for on average ~90% of all neutrophils in both sexes. Already upon emergence from hibernation, these young, immature neutrophils accounted for 92.4% (SD=7.56; n=16) of all neutrophils in males and for 90.0% (SD=5.4; n=6) in females. For males, the number of these young neutrophils were significantly increased during the reproductive period (Model: N_{young} = period + ID random; n=52; ANOVA: p=0.003, MM: p_{Rep}=0.014).
In both sexes body condition had a negative effect on P counts (Tab. A2 & A5), however in males this influence only has a tendency.

3.3. Comparison of WBC parameters among low and high food/reproductive years in males

Within August and September, which is the time when ripe beech seeds are available during years of seed mast, WBC counts of the low and the high food/repro years were comparable (p>0.1; 2012: 13,710±5,718 WBC/µL; n=35; 2013+2014: 12.975±3.710 WBC/µL; n=51). As we had only information on the proportions of different leukocyte types in males and not on their absolute numbers for certain time periods in 2012, we restricted the comparisons among years to the proportions of P and L. The proportions of P (P_{prop}) differed strongly directly after emergence from hibernation, with significantly lower values occurring in the low food/repro year (Modell: ln P_{prop} = $food_{low}$+ID random; n=33; $estimate_{food\ high}$= 0.54; p=0.007; Fig. 2). In some individuals that were sampled shortly after emergence from hibernation, innate immune cells were nearly absent, especially in the low food year. In 2012, 8 out of 10 individuals sampled had less than 3.5% N in the circulation directly after termination of hibernation, whereas such a small proportion of N was only found in 2 individuals out of 23 at the beginning of 2013 and 2014.

To investigate the effect of reproductive investment and food availability, we compared the July P/L_{prop} ratios of sexually quiescent males to sexually active males during the time of mating and furthermore to males under food limitation as well as during the period of high seed mast. In comparison to the sexually quiescent males in July, the P/L_{prop} ratios were significantly elevated in sexually active males during the mating season. Also males under food limitation revealed significantly higher P/L_{prop} ratios, whereas males under high food availability exhibited only a tendency to higher P/L_{prop} ratios (Table A.7, Fig. 3). When comparing these two groups (high versus low food availability), the P/L_{prop} ratios of food limited males were higher, however, this difference was statistically not significant (Fig. 3, p=0.20).

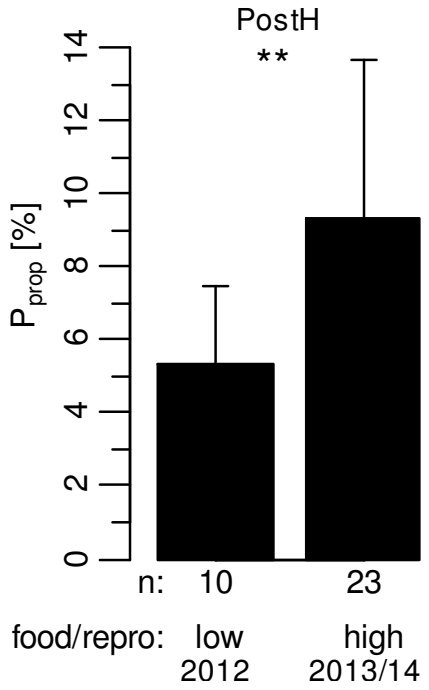

Fig. 2. P proportions in male edible dormice during PostH in low and high food/repro years (low: 2012 vs high: 2013 and 2014; mean and SD).

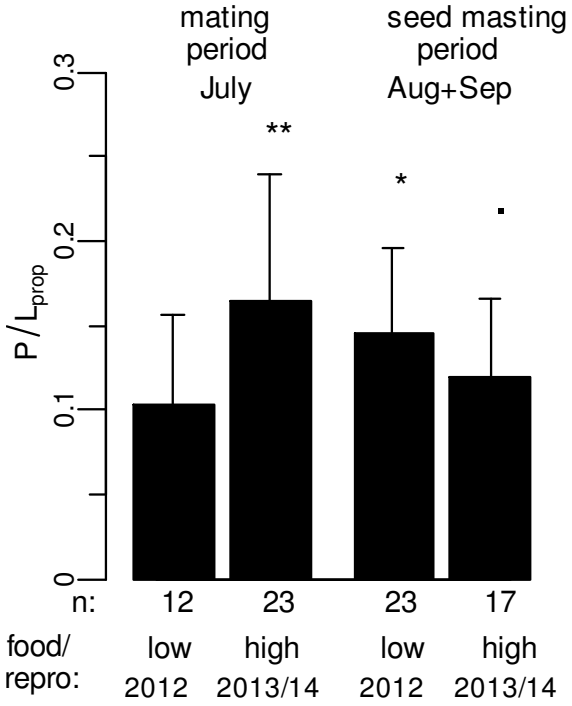

Fig. 3. P/L_{prop} ratio of male edible dormice during the "potential" mating period (left) and the "potential" seed masting period (right) in low and high food/repro years (mean and SD). Note that males in the low food/repro year remained sexually quiescent and lacked high quality food during August (bars one and three). Significant variations among periods were determined in comparison to sexually quiescent males (left bar) as a reference.

3.4. Effects of population density on WBC Parameters

During the periods of high reproductive investment in 2013 and 2014 the P/L ratio of both males and females inhabiting the study site with the highest population density (BG) was lowest compared to those of individuals captured in all other study sites. These differences were most prominent for BS and JH (Table A.8, 9, Fig. 4).

P/L_{prop} ratios measured in non-reproductive males in 2012 during the time period, when mating normally takes place in reproductive years were lowest again in BG, with HE ranging on a comparable level and significant differences in comparison to SF and JH (Table A.10, Fig. 5).

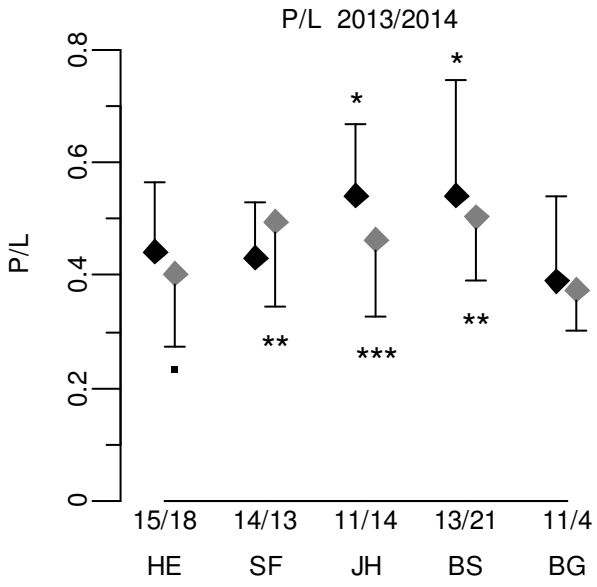

Fig. 4. P/L ratio of male (black) and female (grey) edible dormice inhabiting study sites with markedly different population densities during the period of high reproductive investment in the years 2013 and 2014 (mean and SD). Sample sizes are given for each study site (males/females). Significant variations among study sites were determined in comparison to BG as a reference. Study sites are sorted in ascending order of dormouse population densities (see also Tab. 1).

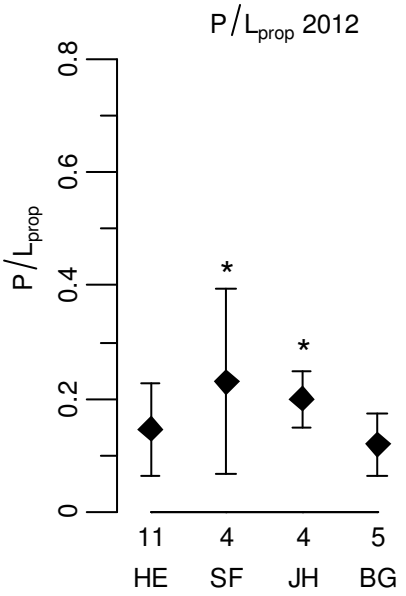

Fig. 5. P/L_{prop} ratio of sexually quiescent male edible dormice inhabiting study sites with markedly different population densities during the potential mating period in the low reproductive year 2012 (mean and SD). Significant variations among study sites were determined in comparison to BG as a reference. Study sites are sorted in ascending order of dormouse population densities (see also Tab.1).

4. Discussion

4.1. Hibernation-induced impairment of the innate immune system lingers after emergence

Edible dormice emerged from hibernation with extremely reduced P (phagocyte= neutrophils and monocytes) counts and proportions that only recovered slowly, whereas L (lymphocytes) counts constituted high proportions and counts throughout the entire active season. An extreme leukopenia and drastically compromised functionalities of basically all immune system components represent the typical epiphenomenon of hibernation, all of which were generally found to revert immediately upon arousal (Bouma et al., 2010; Inkovaara and Suomalainen, 1973; Prendergast et al., 2002; Reznik et al., 1975; Spurrier and Dawe, 1973; Suomalainen and Rosokivi, 1973; Szilagyi and Senturia, 1972; Wenberg et al., 1973). During torpor bouts, leukocytes (up to > 90%) are cleared from the circulation and stored in other body compartments like e.g. the lung, the liver or secondary lymphoid organs, adherence to vessel walls is another thinkable possibility, from where they can be rapidly released into the blood stream upon arousal (Bouma et al., 2013, 2010). Hence, the post-hibernation leukocyte pattern of the innate immune system described in our study differs distinctively from those found so far in all other hibernators investigated. Even without information on the functionality of the present P cells, their reduced numbers strongly suggest that the innate immune system which represents the first line of defense against pathogens is impaired upon and after emergence.

4.2. Hibernation pattern might determine phagocyte death and renewal

During hibernation, an extreme retardation of all kinds of cell processes occurs, which applies to organ and tissue functions, cell growth and mitosis as well as senescence and apoptosis (Adelstein et al., 1966; Breukelen et al., 2010; Brock, 1960; Fleck and Carey, 2005; Kruman, 1992; Kruman et al., 1988a; Kurtz et al., 2006; Zatzman, 1984). In hibernators, that were shown to restore leukocyte numbers immediately after arousal, the major part of neutrophils (N) constituted of mature cells (Bouma et al., 2011; Inkovaara and Suomalainen, 1973; Suomalainen and Rosokivi, 1973; Szilagyi and Senturia, 1972). These findings support two established presumptions: First, the life span of immune cells is substantially prolonged during hibernation, providing that leukocytes survive the extreme physiological conditions of hibernation although the duration of the hibernation period clearly outlasts the normal life span of N and monocytes in an euthermic organism (N: ~12 h, monocytes: ~60 h, in mice, comparable values in other mammals) (Basu et al., 2002; Casanova-Acebes et al., 2014; Eash et al., 2009; Lord et al., 1991; von Vietinghoff and Ley, 2008), whereas antigen-experienced (activated) L usually have longer life spans than the duration of the total hibernation period (Sprent and Tough, 1994). Second, leukocytopoiesis is virtually non-existent during torpor.

The persistent neutro- and monocytopenia in aroused animals of our study species, however, strongly suggests that P stores are largely depleted during hibernation and therefore have to be regenerated. Investigations of leukocyte patterns in hibernators have so far been conducted in the laboratory, i.e. under conditions that are hardly comparable to those hibernators face in the wild and/or carried out on species that either resume feeding during hibernation, like the European hamster, exhibit shorter total hibernation periods or higher arousal frequencies, compared to our study species (Bouma et al., 2010; Suomalainen and Rosokivi, 1973). Edible dormice completely cease feeding during hibernation and hibernate for an extremely long time period (>8 months), with arousals that occur more frequently and are longer-lasting towards the end of the hibernation cycle, in dependency on energy stores (Bieber et al., 2014; Fietz et al., 2005; Vietinghoff-Riesch, 1960). As during torpor mitoses and other cell processes nearly cease and their initiation is basically restricted to the euthermic phases (Carey et al., 2003; Clarke and Fraser, 2004), especially arousals contribute on the one side to senescence of present and on the other side to the generation of new immune cells. Furthermore, it is important to note that the reactivation of various processes occurs only during arousals towards the end of the hibernation cycle (e.g. spermatogenesis, stomach and intestinal epithelial cells) (Kruman, 1992; Kruman et al., 1988a), therefore a restriction of the generation of immune cells to final arousals of the hibernation period is probable and contributes to the overall reduction of energy consumption during hibernation. More frequent arousals consequently trade off maintenance of homeostasis and preparation for the following active season against energy consumption and decreasing half-life of P cells, resulting in apoptosis of the short-living P cells. Our finding that almost exclusively immature and band Ns were present in the blood of edible dormice upon emergence supports the existence of such a trade-off that leads to P death as well as partial restoration during arousals late in the period of hibernation.

Former studies have shown that edible dormice seem to be able to predict future food availability and adjust their energy expenditure accordingly: individuals remain sexually quiescent and in an energy saving mode with frequent and extended hypothermic periods in years of restricted food availability (Bieber, 1998; Bieber and Ruf, 2009; Fietz et al., 2009; Hoelzl et al., 2015; Schlund et al., 2002). We assume that this preparatory energy-saving mechanism also applies to their immune defense. Interestingly, we observed that particularly in 2012, Ps were virtually absent in several individuals captured directly after emergence from hibernation (<3.5%). Accordingly, the low proportion of Ps at the beginning of the low food/repro year 2012 indicates that the extent of preparatory investment into immune defense represents an adjustment to limited food availability and reproductive skipping in the subsequent active season. We therefore assume that arousal frequency and their specific use for preparatory processes, including restoration of immune cells,

might be highly flexible, depending on the trade-off between energy status and prospective environmental conditions.

4.3. Energetic trade-offs impair the recovery of innate immune cells

Surprisingly, after termination of hibernation the restoration of P counts in edible dormice took about 4 weeks, although the principle haematopoietic cytokines for phagocytes, G-CSF, GM-CSF and IL-3 should be able to restore cell numbers much faster (14-fold N increases within a few days) (Lieschke et al., 1994; Lord et al., 1991). Different mechanisms could cause this slow recovery of detectable P in peripheral blood. First, a strong margination of P to vessel walls during PostH could lead to reduced numbers. Second, P cells might be generated, but retained in the bone marrow. Third, energetic constraints might restrain haematopoiesis on a reduced level for considerable time after emergence. In other hibernating species (mature) N were equally present after hibernation (Bouma et al., 2011; Inkovaara and Suomalainen, 1973; Suomalainen and Rosokivi, 1973; Szilagyi and Senturia, 1972), making a strong PE vessel wall adherence of N in dormice rather unlikely. Furthermore, in our case, circulating Ns constituted almost exclusively of immature cells after the hibernation period and their numbers constantly increased throughout PostH and Rep. However, immature Ns would assumably not be that dominant if a strong vessel wall margination or retention of P cells in the bone marrow would have caused low numbers for considerable time. Instead, in our opinion several findings support the third possibility. When emerging from hibernation, edible dormice have lost about 30% of their pre-hibernation body mass and are confronted with a still restricted food availability (Fietz et al., 2005). At the same time, the reestablishment of various organs, such as the digestive system and gonads induce high energetic costs (Carey, 1995; Christian et al., 1972; Darrow et al., 1988; Fietz et al., 2004; Hume et al., 2002; Kenagy and Barnes, 1988; Lee et al., 1990). This also applies to the reactivation of the immune system. Our results strongly suggest that after hibernation edible dormice allocate only a limited amount of energy into the immune system but rather invest into other body functions. The depleted P store in connection with the long-living Ls necessitate a shift of investment particularly into granulopoiesis. However, reactivation of granulopoiesis occurs only delayed / an accelerated granulopoiesis to restore P cells occurs only delayed /production of P occurs delayed, consequently resulting in an impaired immune defense, since innate immune cells form the first line of defense in any infection. In contrast to the assumption that seasonal nadirs in immune competence may help to survive demanding and stressful periods (Martin, 2009), this delayed investment in edible dormice seems to impair fitness, as the mortality rate of this species is highest directly after emergence from hibernation (Lebl et al., 2011). Thus, an energetic trade-off before and upon emergence from hibernation seems to

compromise immune defense and therefore account for the high mortality rates of dormice in spring.

4.4. Alterations in the P/L ratio indicate stress associated with reproduction and food limitation

Whereas the augmentation in the P/L ratio after hibernation is exclusively caused by recovering P numbers, we first observe a physiological stress response during Rep, when in both sexes a conjoint increase in P and decrease in L counts occur. Our analyses reveal that Ns as well as the low number of monocytes increase during the reproductive season. In females, the rise in the P/L ratio was even more pronounced during pre-hibernation, when high maternal investment during late lactation coincides with pre-hibernation fattening. In line with these observations P/L_{prop} ratios measured in reproductive males during the mating season were significantly elevated in comparison to those measured in sexually quiescent males during the same time period in the low food/repro year. These results clearly reveal that reproductive activities impose chronic stress associated with physiological implications in both sexes. Results of another study carried out on edible dormice of the same populations reveal that high GC levels in the urine occur during periods of reproductive activity as well as food limitation (Havenstein et al. unpubl data), which is perfectly in accordance with changes in the P/L ratio presented here. Overall, we detected a negative correlation between P counts and body condition in both sexes. On the one hand, animals with a higher body condition might have invested more into the restoration of P cells already before termination of hibernation (see section 4.2), implying that less P cells need to be generated thereafter. On the other hand, this may indicate that individuals in low body condition are more vulnerable to infections and therefore frequently exhibit higher P numbers during stressful periods as a protective measure (Johnstone et al., 2012b) whereas those with high body condition are more resilient to stress, e.g. because they can rely on their energy reserves to quickly generate large numbers of P cells upon pathogen encounter. Recent research has drawn much attention to metabolic hormones as modulators of immune function (Carlton et al., 2012). Accordingly, leptin regulates energy homeostasis and is also thought to influence haematopoiesis (Claycombe et al., 2008), which might mediate the observed relationship between body condition and P cell number. Summarized, more research is needed to explain this relationship. Furthermore, especially in males it becomes obvious that the decline in L counts started time-shifted, belated to the onset of the stressful reproductive period and still kept diminishing after the mating season. This stands in contrast to findings of attenuation of L trafficking when stress becomes chronic (Dhabhar and McEwen, 1997), but is in line with the findings that L apoptosis increases after prolonged stress and persists even after the stressor has ended (Nagata and Golstein, 1995; Penninger and Mak, 1994; Shi et al., 2003). Accordingly, our results support the assumption that stress has to persist for a prolonged time before it manifests in alterations of the P/L ratio (Davis

et al., 2008; Johnstone et al., 2012a). Furthermore, the persistent high P/L ratio after the reproductive period, denotes that regeneration does not occur immediately after the stressful situation is terminated but requires time for recovery. Dhabhar (2009) strongly suggested that stress-induced changes of blood leukocyte populations represent an enhancement of immunity at sites of potential pathogen encounter. However, it is known from various investigations that chronic stress eventually also induces immunosuppressive effects (Glaser and Kiecolt-Glaser, 2005). Based exclusively on the present information on the P/L ratios it is hard to decide whether alterations in the WBC picture constitute advantageous, immunoenhancing or disadvantageous, immunosuppressive changes. However, survival probabilities in edible dormice are distinctively reduced in years of high reproductive investment demonstrating that dormice incur high reproductive costs (Lebl et al., 2011; Ruf et al., 2006). Accordingly, the strong and persistent elevations in the P/L ratios detected here rather indicate that the threshold to adverse, immunosuppressive effects of chronic stress has been exceeded and impair future resilience.

Food shortage also represents a stressful condition and in our study P/L_{prop} ratios of the low food year were significantly higher in August/September (when ripe seeds are available in a high food year) than in July. Interestingly, when comparing males of low with males of high food years during the period of seed mast, the ratio was higher in food limited males, but the difference was not very pronounced. Furthermore, males in the seed masting period of a high food/repro year exhibited still slightly elevated P/L_{prop} ratios compared to their non-reproducing conspecifics in July of a low food/repro year. These findings may be due to the above described delayed recovery of leukocyte alterations after the reproductive stress has ended in reproductively active males and therefore the elevated P/L ratio still persists under high food availability (see above). However, compared to the high P/L_{prop} ratio found during mating, the P/L_{prop} ratio elevation during the period of mast failure was moderate. From the functional perspective, a poorer body condition is associated with an elevated P/L ratio due to an adaptive response to a more risky environment or life stage as these animals are more prone to injury and infection (Johnstone et al., 2012b). Even though we agree that this relationship probably also applies in our case, we furthermore assume that increased GC levels, that cause elevated P/L ratios, may represent an adaptive metabolic response that helps to mobilize energy. However, as edible dormice skip reproduction in years of low food supply, food limitation results in a fitness trade-off in terms of reduced reproductive output in favor of survival.

4.5. P/L ratio does not correlate with high population densities

We assumed that high population densities might amplify stressful situations associated with intraspecific competition for resources and mating opportunities. In contrast to our expectations, the P/L ratio was always lowest in the study site with the highest population density (BG). Thus,

population densities reached in our study sites do obviously not produce adverse effects in respect to the leukocyte picture, even during periods of high reproductive investment. Results rather indicate that individuals from this study site experienced least stress which might even explain high dormouse abundances. Furthermore, P/L and P/L_{prop} ratios of individuals from the study site with the lowest population density (HE) ranged on comparably low levels, suggesting that factors other than population density trigger the level of experienced stress in edible dormice. In snowshoe hares, predation risk causes high stress levels and drastic declines in population densities (Sheriff et al., 2009). Accordingly, habitat-specific variations in predation pressure may be a possible cause for the detected differences in the physiologic stress response as well as for the differences in population densities. Studies in other small mammals indicate that HPA-axis functioning is altered in varying degrees by reproductive effort and food scarcity, which may have major influences on fitness (Boonstra et al., 2014). Thus by assessing potential stressors and their impact on the life history of the edible dormouse habitat-specific differences in survival and reproduction might be further unraveled.

5. Conclusions

The leukocyte differential of dormice composes to a great extent of Ls and a small proportion of Ps. This is in line with expectations for a slow-living species that should invest stronger into the specific immune defense while reducing constitutive and inflammatory immunity to diminish auto-immune damage and establish a strong immunity against recurrent pathogens. Results of our study further demonstrate that the leukocyte blood picture is highly dynamic and responds sensitively to physiological conditions associated with hibernation, reproduction as well as food shortage. The functional significance of changes in the leukocyte picture derives from the clear link of reduced survival rates to elevated stress levels indicated by high P/L ratios as well as reduced numbers of phagocytes upon emergence, which form the first line of defense in any infection. Future environmental conditions seem to influence preparatory mechanisms occurring during arousals in hibernating individuals, and determine the number of these innate immune cells at the beginning of the active season. Energetic constraints seem to play a central role for leukocyte picture variations, implying trade-offs between the immune system and other competing body functions. Findings of our study reveal that the edible dormouse represents an excellent model organism to investigate regulatory mechanisms of the immune system under natural conditions.

Author contributions

NH and JF designed experiments, performed research, analyzed data and wrote the manuscript. FL performed research. VS assisted in writing the manuscript.

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Appendix Chapter II

Table A.1. Estimates of fixed effects of the general linear mixed model for P counts of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: sqrtP = intercept + period + body condition + time of day + ID random, n=166

sqrt P		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		128.087	1	<0.001	2.531	0.224	103	11.318	<0.001
Period PE		23.462	3	<0.001					
	R				0.161	0.076	57	2.121	0.038
	Rep				0.287	0.061	57	4.734	<0.001
	PreH				0.222	0.071	57	3.130	0.003
Body condition		3.310	1	0.069	-0.115	0.063	57	-1.819	0.074
Time of day		14.984	1	<0.001	-0.051	0.013	57	-3.871	<0.001

Table A.2. Estimates of fixed effects of the general linear mixed model for L counts of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: sqrt L = intercept +period + ID random, n=166

sqrt L		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		1020.013	1	<0.001	3.185	0.100	102	31.938	<0.001
Period R		16.027	3	0.001					
	PE				-0.078	0.125	61	-0.626	0.534
	Rep				-0.235	0.112	61	-2.095	0.040
	PreH				-0.424	0.122	61	-3.480	0.001

Table A.3. Estimates of fixed effects of the general linear mixed model for the P/L ratio of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: sqrt P/L = intercept + period + time of day + ID random, n=166

sqrtP/L		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		254.287	1	<0.001	0.807	0.051	103	15.946	<0.001
Period PE		53.651	3	<0.001					
	R				0.034	0.024	58	1.384	0.172
	Rep				0.122	0.019	58	6.281	<0.001
	PreH				0.125	0.021	58	5.877	<0.001
Time of day		30.910	1	<0.001	-0.023	0.004	58	-5.560	<0.001

Table A.4. Estimates of fixed effects of the general linear mixed model for phagocyte P counts of female edible dormice s collected in all study sites during the entire active period of 2013 and 2014. Model: sqrt P = intercept + period + body condition + ID random, n=169

sqrt P		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		148.420	1	<0.001	2.144	0.176	102	12.183	0.000
Period PE		19.506	3	<0.001					
	R				0.201	0.093	67	2.168	0.034
	Rep				0.347	0.093	67	3.747	0.000
	PreH				0.441	0.105	67	4.190	0.000
Body condition		7.490	1	0.006	-0.178	0.065	67	-2.737	0.008

Table A.5. Estimates of fixed effects of the general linear mixed model for L counts of female edible dormice collected in all study sites during the entire active period of 2013 and 2014. Model: lnL = intercept + period + study site + ID random, n=169

ln L		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		799.810	1	<0.001	2.413	0.085	98	28.281	<0.001
Period R		17.177	3	0.001					
	PE	10.771	4	0.029	-0.161	0.084	68	-1.916	0.060
	Rep				-0.220	0.074	68	-2.961	0.004
	PreH				-0.288	0.070	68	-4.085	<0.001
Study site BG		10.771	4	0.029					
	BS				-0.127	0.094	98	-1.354	0.179
	HE				0.062	0.094	98	0.661	0.510
	Jh				-0.153	0.094	98	-1.631	0.106
	SF				-0.192	0.098	98	-1.950	0.054

Table A.6. Estimates of fixed effects of the general linear mixed model for the P/L ratio of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: sqrt P/L = intercept + period + study site + time of day + ID random, n=169

sqrt P/L		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		76.944	1	<0.001	0.653	0.074	95	8.772	<0.001
Period PE		42.025	3	0.000					
	R				0.025	0.030	65	0.840	0.404
	Rep				0.079	0.029	65	2.715	0.009
	PreH				0.160	0.031	65	5.165	<0.001
Study site BG		14.605	4	0.006					
	BS				0.081	0.031	95	2.605	0.011
	HE				0.050	0.031	95	1.625	0.107
	Jh				0.105	0.031	95	3.389	0.001
	SF				0.097	0.032	95	3.019	0.003
Time of day		4.545	1	0.033	-0.013	0.006	65	-2.132	0.037

Table A.7. Estimates of fixed effects of the general linear mixed model for the P/L_{prop} ratio of male edible dormice collected in all study sites except BS during the (potential) mating periods (July) and the (potential) seed masting periods (August + September) in the low food/repro year 2012 and the high food/repro years 2013 and 2014. Model: sqrt P/L_{prop} = intercept + condition (food/repro) + study site + ID random, n=75

sqrt P/L_{prop}		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		114.226	1	<0.001	0.257	0.030	59	8.636	<0.001
Condition $Repro_{low}$		13.709	3	0.003					
	$Repro_{high}$				0.104	0.030	9	3.494	0.007
	$Food_{low}$				0.084	0.029	9	2.903	0.018
	$Food_{high}$				0.056	0.032	59	1.750	0.085
Study Site BG		9.182	3	0.027					
	HE				0.040	0.023	59	1.764	0.083
	KW				0.079	0.029	59	2.739	0.008
	JH				0.068	0.034	59	2.003	0.050

Table A.8. Estimates of fixed effects of the general linear mixed model for the P/L ratio of males collected in all study sites during the periods of high reproductive activity of 2013 and 2014. Model: sqrt P/L = intercept + population density + time of day + ID random, Season 3, m, n=64

sqrt P/L		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		156.70	1	<0.001	0.972	0.078	42	12.518	<0.001
Population density BG		12.582	4	0.014					
	BS				0.093	0.038	42	2.464	0.018
	HE				0.050	0.037	42	1.358	0.182
	JH				0.095	0.040	42	2.387	0.022
	SF				0.001	0.038	42	0.037	0.971
Time of day		24.501	1	<0.001	-0.031	0.006	16	-4.950	<0.001

Table A.9. Estimates of fixed effects of the general linear mixed model for the P/L ratio of females collected in all study sites during the periods of high reproductive activity (periods 3 + 4) of 2013 and 2014, P/L was square root transformed. Model: sqrt P/L = intercept + Julian day + population density + time of day + ID random, Season 3+4, n=112

sqrt P/L		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		2.388	1	0.122	0.253	0.164	67	1.545	0.127
Julian Day		15.711	1	<0.001	0.002	0.001	38	3.964	<0.001
Population density BG		13.470	4	0.009					
	BS				0.138	0.047	67	2.938	0.005
	HE				0.094	0.047	67	1.971	0.053
	JH				0.162	0.047	67	3.432	0.001
	SF				0.124	0.046	67	2.675	0.009
Time of day		6.233	1	0.013	-0.019	0.008	38	-2.497	0.017

Table A.10. Estimates of fixed effects of the general linear mixed model for the P/L ratio of males collected in HE, SF, JH, and BG in 2012 during the period equivalent to Rep in a reproductive year. P/L was square root transformed. Model: sqrt P/L = intercept + population density + ID random, n= 24

sqrt P/L_{prop}		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		183.106	1	<0.001	0.350	0.026	37	13.532	<0.001
Population density BG		8.311	3	0.040					
	HE				0.033	0.036	37	0.916	0.366
	JH				0.108	0.051	37	2.128	0.040
	SF				0.104	0.043	37	2.420	0.021

Chapter III: The oxygen delivery system

Life History Written in Blood: Erythrocyte Parameters in a Small Hibernator, the Edible Dormouse

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Abstract

The oxygen delivery system is one major determinant of the performance of vertebrates and responds sensitively to a variety of internal and environmental factors. To understand physiological mechanisms underlying variations of fitness, we investigated effects of demanding conditions associated with certain life history events, food availability, and population density on the oxygen delivery system in free-ranging edible dormice (*Glis glis*). We therefore sampled blood (n=248) and urine (n=319), performed an erythrocyte haemogram and visually determined the presence of haemoglobinuria. Reproduction leads to increased mortality in edible dormice and our study now revealed severe haematological impairments during reproduction that were associated with nutrient and energy deficits and stress. These effects were even more pronounced in subsequent reproductive years, indicating prolonged physiological impairment. Under limited food availability the rate of erythrocyte generation was reduced. This seems to be part of an energy saving strategy instead of representing a poor body condition as survival probability in this species is high in years of low food availability. A high prevalence ratio of haemoglobinuria (up to 85 %) at the end of the active season indicated amplified erythrocyte destruction through haemolysis. This may be the result of a preparative mechanism to avoid massive oxidative damage during the long hibernation period. Most

ecophysiological studies so far focus on single erythrocyte parameters on a short time scale, which could be misleading. Our results clearly highlight that a wide-array RBC approach is a powerful tool for investigating mechanisms underlying physiological performance and fitness, also for other vertebrate taxa.

1. Introduction

The examination of individual physiological responses to alterations in environmental conditions may provide insights into mechanisms underlying population declines. Suitable targets of investigation are functions that are vital for the whole organism, like e. g. metabolism, endocrine regulation, and immunity (Cooke et al., 2013; Wikelski and Cooke, 2006) but also the delivery of oxygen from the lungs to body tissues by the highly specialized RBCs (Keohane et al., 2015). The oxygen delivery system is vulnerable to a variety of internal and environmental pathophysiological factors, which can confound the balance between erythrocyte generation and destruction (Hoffman et al., 2013). Reduced RBC mass and anaemia may severely impair body condition and the performance of an organism, resulting in fatigue, dyspnea, cardiovascular and neurological complications (Lipschitz, 2003; Tyler and Cowell, 1996). In the bone marrow, erythropoiesis is prone to disturbance under conditions of irradiation, malnutrition and fasting, as well as disturbances of hormones involved in haematopoiesis (Hoffman et al., 2013; Koury and Ponka, 2004; Tyler and Cowell, 1996). In the circulation, RBCs are vulnerable to damages that shorten their lifespan, such as mechanical rupture due to shear forces, energy depletion, chemical intoxication, osmotic and oxidative stress, the latter being the most common form of accelerated RBC aging and destruction (Lang et al., 2005; Lutz and Bogdanova, 2013; Sivilotti, 2004). Physical activity and stress affect the oxygen delivery system in several ways: strong exercise increases RBC aging and damage and is known to reduce the haematocrit through increasing the plasma volume (Mairbäurl, 2013; Santos-Silva et al., 2001; Smith, 1995). High stress hormone levels are associated with an exacerbation of oxidative stress (Costantini et al., 2011; Jarolim et al., 1990; Oishi et al., 1999; Orzechowski et al., 2002; Sivilotti, 2004), and a downregulation of the protective antioxidant systems (Orzechowski et al. 2002), resulting in enhanced haemolysis and reduced RBC life span while simultaneously boosting the release of RBCs from the bone marrow into the blood (Fisher and Crook, 1962; Lodish et al., 2010) which may help to recover from anaemia. Accelerated release of RBCs frequently results in the state of a regenerative anaemia, characterized by an elevated liberation of reticulocytes, large and immature erythrocytes that are less capable to load oxygen (Aslinia et al., 2006).

Several studies have used haematological parameters to evaluate the effects of habitat quality, age,

pathogen load, seasonality or stress on health and body condition in different mammalian species, however, they mostly concentrated on RBC counts, haematocrit or haemoglobin, possibly in conjunction with other blood parameters or investigated the impact of only a single factor (Beldomenico et al., 2008; Boonstra et al., 2001; Gilot-Fromont et al., 2012; Jégo et al., 2014; Mira and Mathias, 1994; Ots et al., 1998; Sealander, 1964). As each organism is faced with a variety of challenges at the same time, single parameters like the haematocrit can be equivocal (Mairbäurl, 2013) and as disturbances can have varied physiological causes (Hoffman et al., 2013; Tyler and Cowell, 1996), the investigation of a more comprehensive RBC picture under consideration of several potential effectors helps to identify mechanisms underlying variations in health and causes of population declines in detail (Johnstone et al., 2015).

Our study species, the edible dormouse (*Glis glis*) is a small arboreal rodent characterized by an extraordinarily long hibernation period of up to 8 months (Bieber et al., 2014; Fietz et al., 2005). In edible dormice, as in other hibernators, heart and metabolic rates are drastically diminished during hibernation, accompanied by a strongly reduced T_b often approaching ambient temperature (Bieber et al., 2014; Ruf and Geiser, 2015). Edible dormice are highly synchronized in their yearly cycle (Fietz et al., 2009), but populations differ distinctively concerning body mass and population densities (Fietz and Weis-Dootz, 2012). Caused by the irregular seed production of their main feeding tree species, the European beech (*Fagus sylvatica*), this small rodent is confronted with extended periods of food scarcity, which accounts for sexual quiescence of whole populations during years of mast failure and high reproductive activity in full mast years (Lebl et al., 2011; Ruf et al., 2006). We further found elevated stress hormone levels during reproduction in both sexes (Havenstein et al., unpublished data), that led to stress-induced alterations in immune-cell counts (Havenstein et al., 2016) and were associated with previously determined reduced survival probabilities (Lebl et al., 2011; Ruf et al., 2006).

The aim of our field study was to elucidate the effects of stressful and demanding situations like hibernation, reproductive activity, variations in food availability and high population density on the oxygen delivery system in free-ranging edible dormice to understand physiological mechanisms underlying variations of fitness parameters, i.e. survival and reproduction. We therefore sampled blood and urine of edible dormice between 2012 and 2014 in five different study sites in South Western Germany, performed a haemogram and visually determined the presence of haemoglobinuria.

2. Material and Methods

2.1. Study animal & Study Sites

The arboreal edible dormouse (*G. glis*) is the largest European dormouse with a body mass of around 100 g (Schlund, 2005). In Central Europe, this nocturnal rodent occurs preferentially in deciduous mixed forests dominated by European beech (*Fagus sylvatica*; Schlund 2005). In Germany, this obligate hibernator spends approximately 8 months in underground burrows, generally from the end of September until the end of May (Vietinghoff-Riesch, 1960), mate in July and produce one litter per year. After a gestation period of about 30 days, litters averaging 5-6 young are born in August (Fietz et al., 2009). For a small mammal, edible dormice are comparatively long-lived and may reach ages of up to 12 years with an average longevity of 3-4 years in nature (Ruf et al., 2006). During the warm season, dormice frequently use nest boxes to rest during the day and to rear their offspring, which makes them easily accessible for scientific studies. This study was conducted at five different sites located in mixed deciduous forests in south western Germany. In each study site, nest boxes were installed three meters above ground at the intersections of grid lines marking 30×30 m squares (Tab. 1, for details see Tab. 1 and Fietz et al., 2014).

Table 1. Locations including coordinates, sizes of forest and study sites, total number of individuals captured per hectare between 2012 and 2014.

Study site	Locations near the city	Coordinates	Elevation [m]	Forest size [ha]	Size of study site [ha]	Number of nest boxes	Number of individuals captured in 2012 - 2014 (individuals/ha)
HE	Tübingen	48°33’03.38’’N 8°59’59.72’’E	496	15,000	20.4	228	14.1
KW	Ulm	48°22’56.50’’N 9°55’54.45’’E	595	135	7	70	18.7
BG	Ulm	48°25’22.36’’N 9°57’43.17’’E	612	70	7	70	64.9
BS	Ulm	48°29’21.18’’N 9°58’219.02’’E	606	33	7	70	41.1
JH	Ulm	48°22’11.13’’N 9°49’3.44’’E	633	11	7	70	26.6

2.2. Capture-mark-recapture & Measurements

We checked the nest boxes at all study sites during daytime at bi-weekly intervals from the end of May through September in 2012 to 2014. Upon first capture, we marked each individual with a transponder (Trovan, EURO I.D. Usling, Weilerswist, Germany). We recorded sex, body mass using a 300 g spring balance (Pesola, Baar, Switzerland; division: 2 g, accuracy: 99.7%), measured tibia length and in males testes length to the nearest 0.1 mm using a sliding caliper. Tibia length was used as a proxy for body size. Body condition was defined as body mass (g) divided by tibia length (mm). Reproductive activity was determined in males by checking for the presence of tangible testis. Females that were captured lactating, or showed clear signs of gestation or former lactation were classified as reproductive for the respective study year.

Blood was collected into EDTA-coated tubes (Sarstedt, Microvette 200) by punctuation of the vena facialis of either the left or right side with a hollow/23-gauge needle. During 2012 we exclusively sampled blood of adult males, whereas in 2013 and 2014 blood samples were collected in both sexes. Samples were stored for maximally 8 hours in a cool bag until processing. Urine was sampled between 2012 and 2014 in both sexes directly into 1.5 ml tubes whenever an animal was urinating during the handling procedure and stored at -20°C.

2.3. Laboratory parameters

Red blood cell count (RBC /µL), haematocrit (Hct in %), haemoglobin concentration (Hb in g/dL), mean corpuscular volume (MCV in fL), mean corpuscular hemoglobin (MCH in pg), mean corpuscular hemoglobin concentration (MCHC in g/dL), red blood cell distribution width (RDW in fL , based on the width of the red cell distribution curve at the 20 % height level) were obtained of blood samples collected in 2013 and 2014 with a haematology system (pocH-100i, Sysmex, Kobe, Japan) using 12 µl blood. In 2012 RBC, Hct, Hb, MCV, MCH and MCHC were obtained from blood samples between 30th of July until the end of the active season on a scil Vet abc (Viernheim, Germany) using 18 µl of blood.

We assessed urine color after centrifugation according to RAL colours (http://www.ralcolor.com); from bright to dark: ivory (1014), sand yellow (1002), ocre yellow (1024), beige brown (8024), chocolate brown (8017), black brown (8022). All samples classified as beige brown, chocolate brown and blackbrown clearly notify haemoglobinuria (Keohane et al., 2015) and the prevalence ratio of samples appendent to these classes were considered to monitor intravascular haemolysis.

According to the biology and physiology of the edible dormouse, we defined three distinct main periods during the active season: the post-hibernation period (PostH), the reproductive period (Rep) and the pre-hibernation period (PreH). PostH starts directly when the first animal was captured after the termination of hibernation at the end of May and lasts until the reproductive periods start. As

the timing of the main investment into reproduction differs between males and females, the reproductive season is time-shifted among sexes. Rep in males is represented by mating activities and the presence of well developed testes (Fietz et al. 2004) which last from the beginning of July until mid-August (6th of July until 18th of August), whereas in females this period refers to the period of gestation and lactation lasting from around 20th of July until the end of August. PreH, finally, starts after the termination of reproduction and ends with immergence into hibernation (mid-September) and serves for the accumulation of fat reserves. In females, PreH partly coincides with the raising of the young.

Due to the previously detected pronounced effect of hibernation on immune cells after termination of hibernation (Havenstein et al., 2016), we sought to examine a possible effect of hibernation on the oxygen delivery system. Therefore, we compared RBC parameters of a shortened PreH period ($PreH_{short}$) 2013, between the 2nd of September until the start of hibernation with the consecutive $PostH_{short}$ values of 2014, between the emergence and the 23rd of June. This way, individuals were sampled on their very last days before and the first days after the hibernation period, respectively.
To investigate the effect of food availability on haematology, we compared values of males achieved during the period of ripe seeds (August & September) and when reproduction had ended in high food and reproductive years with values obtained during the same time period in a year of mast failure (2012). However, this analysis was not performed in females as the availability of high quality food coincides with the period of lactation. Therefore, it is impossible to disentangle the pure effect of food availability from effects of high reproductive investment.

2.4. Statistical analyses

We calculated linear mixed effects analyses of the relationships between haematological parameters and biologically relevant variables using R 3.3.1 (R Development Core Team, 2012) and the package *nlme* (Pinheiro et al., 2016) and entering ID as a random factor to correct for repeated measurements. Additionally, we ran an ANOVA with the package *car* (Fox and Weisberg, 2010). We included the following fixed effect variables into the models: "period" (three levels: PostH, Rep, PreH), "study site" (five levels: HE, SF, JH, BS, BG), "body condition", "year" (2013, 2014), “time of day”, “handling time”, "food/repro". Note that in edible dormice reproduction is synchronized with the masting pattern of the beech. In this study the factor "food/repro" can adopt two levels: high versus low. High means that food availability is high and virtually all dormice reproduce within this year (2013 and 2014), low on the other side means, that beeches and dormice completely fail
ed to reproduce during the respective year (2012). As time of day and sampling duration were never significant, we always omitted these variables from the final models. If the variables "study site", "body condition", and "year" were non-significant, the minimisation of the Akaike's Information

Criterion (AIC) determined whether these variables were excluded from the final model. Even though we had a comparatively large sample size for each sex, by including interaction terms into the linear model, the number of terms would have been too numerous. We therefore restricted predictor variables to the main effects. Data were transformed if necessary to achieve normality and homoscedasticity in the mixed model and were assured by visual inspection of histograms and plots of fitted values against residuals. The functions *summary* and *Anova (type 3)* were used to obtain results of the models. Test results were considered significant, if p was <0.05, p-values between 0.05 and 0.1 were considered as a trend. Details of the particular models used are given within the respective results section. We performed Chi-squared tests to analyze effects of sex or season on the prevalence of haemoglobinuria. In Figures, asterisks are used to display significance levels according to linear mixed models with the following indications: "**.**" p<0.1; "*" p<0.05; "**" p<0.01; " ***" p<0.001

3. Results

3.1. Blood samples collected

We collected blood samples of in total 248 adult edible dormice from 2012 until 2014 between May and September in five different study sites. 40 adult males were sampled in 2012, a year of reproductive failure in edible dormice and low beech seed masting. During 2013 and 2014, years of high beech seed production and high dormouse reproductive activity, 103 females and 105 males were sampled. Sample sizes were evenly distributed among study sites.

3.2. Seasonal variations in RBC parameters during the high food/reproductive years 2013 and 2014

In males, most erythrocyte parameters examined showed a clear seasonal pattern with a peak or nadir occurring during reproductive activity. Accordingly, when analyzing the seasonal variation of the RBC picture with data of the PostH period as reference, we detected significant decreases in RBC counts, Hb and MCHC as well as significant rises in MCV, MCH and RDW during the mating season (Tables A1-7; Fig. 1). During the consecutive PreH period, most erythrocyte parameters seemed to revert, but not all of them reached premating values. In detail, during PreH, RBC, Hb, Hct were significantly lower or showed a tendency towards lower values, whereas MCV and MCH were significantly higher compared to PostH values. After the nadir during Rep, the MCHC reached even higher levels during PreH compared to premating values (Tables A1-7, Fig. 1).

During late gestation and early lactation, RBC counts, Hb and Hct in females had a nadir and rose again thereafter, but remained on lower levels during PreH compared to PostH. In this context, RBC

counts and Hb in females exhibited the same seasonal patterns as those found in males. The MCV, MCHC and MCH increased during Rep and remained on high levels during PreH, with highest MCV values during PreH. No significant seasonal variation in the RDW could be detected in females (Tables A8-13, Fig. 1).

The investigation of the effect through the study year (2013 vs. 2014) revealed that in PostH 2014, females exhibited higher Hct, MCV, and RDW, as well as a lower MCHC compared to PostH 2013 (Table 2, A32-38). During Rep in 2014, males showed lower levels in RBC counts, Hb, and Hct, combined with higher levels in MCHC and MCH (Table 2, A26-31), compared to Rep 2013.

Table 2. Female PostH and male Rep values of haematological parameters in the two reproductive years 2013 and 2014. Significant differences according to the linear mixed models are marked by asterisks.

	PostH females					Rep males				
	2013		2014			2013		2014		
	mean	SD	mean	SD		mean	SD	mean	SD	
RBC	9.966	0.862	10.918	0.973		10.299	0.963	9.087	0.995	***
Hb	15.441	1.363	16.796	1.194	.	15.770	1.599	14.700	1.360	**
Hct	48.638	6.604	54.988	4.357	*	53.761	5.049	47.850	4.613	***
MCV	49.384	1.491	50.438	1.901	**	52.241	1.608	52.763	1.958	
MCHC	31.400	1.012	30.581	1.050	*	29.320	0.893	30.744	0.694	***
MCH	15.503	0.529	15.427	0.711		15.307	0.654	16.213	0.598	***
RDW	27.113	1.602	29.119	3.107	**	31.511	2.769	30.788	2.533	

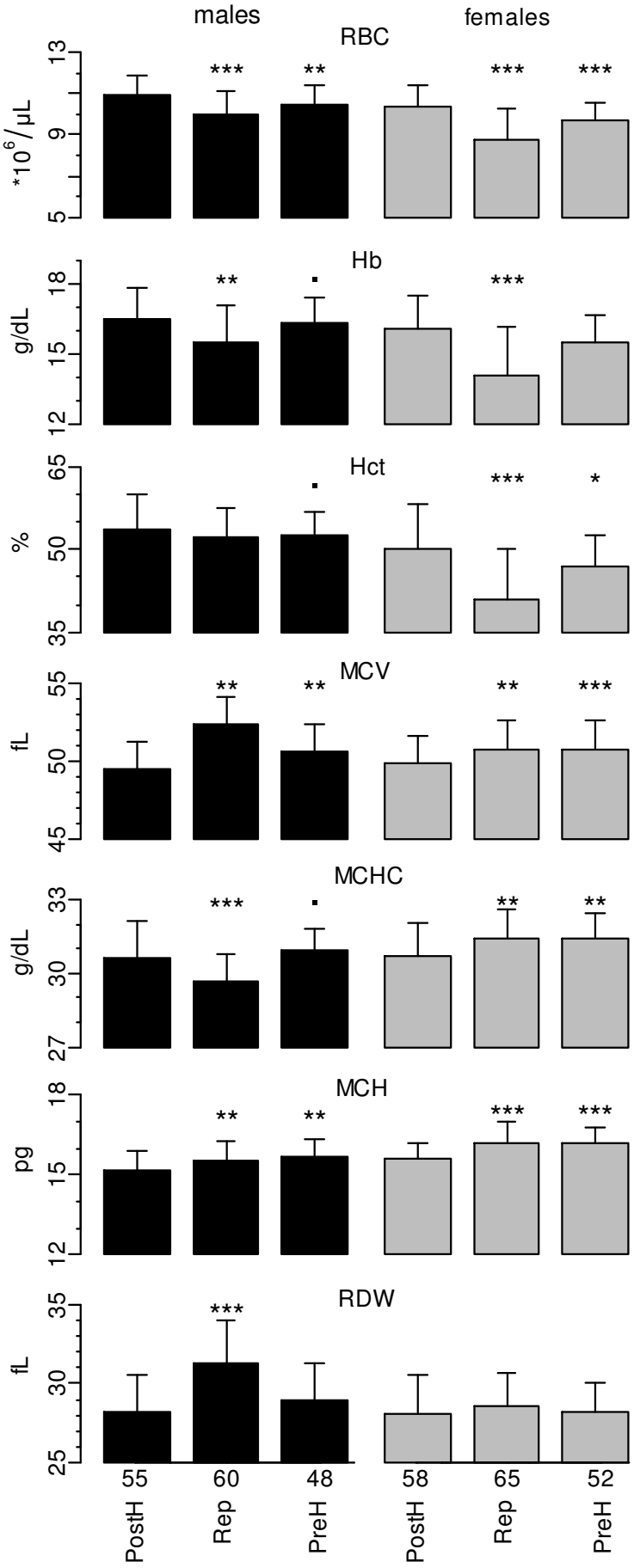

Fig. 1 Seasonal changes in RBC, Hb, Hct, MCV, MCHC, MCH, and RDW of male (left) and female (right) edible dormice during PostH, Rep, and PreH of their active season in the high food/reproductive years 2013 and 2014 (mean and SD). Changes were determined for Rep and PreH in comparison to PostH. Sample sizes are quoted below the bar labels.

3.3. The RBC picture before and after hibernation

In males as well as in females, we detected significantly higher RBC counts, Hb and Hct shortly after the hibernation period $PostH_{short}$ (2014) compared to the preceding $PreH_{short}$ (2013) values. In males, these alterations were accompanied by a decrease in MCH (p=0.08), and in females, the MCH and RDW were significantly higher whereas the MCHC was significantly lower $PostH_{short}$ compared to $PreH_{short}$ (Tables 3, A14-18; models females: hibernation period $PreH_{short}$ + ID random, n=37, DF=34; RBC: Estimate=1.42, p=0.002; Hb: Estimate=1.36, p=0.014; Hct: Estimate=8.12, p<0.001; $1/MCH^2$: Estimate=-0.001, p=0.05; $MCHC^2$: Estimate=-306.02, p<0.001; 1/RDW: Estimate=-0.004, p=0.003).

Table 3. $PreH_{short}$ (2013)- and $post_{short}$ (2014)-hibernation values of male and female haematological parameters. Significant differences according to the linear mixed models are marked by asterisks.

	males					females				
	$PreH_{short}$ 2013 (n=24)	SD	$PostH_{short}$ 2014 (n=16)	SD		$PreH_{short}$ 2013 (n=30)	SD	$PostH_{short}$ 2014 (n=7)	SD	
RBC	10.471	0.883	11.433	0.727	*	9.719	0.935	11.141	1.165	**
Hb	16.433	1.045	17.381	0.991	*	15.427	1.186	16.729	1.341	*
Hct	53.475	3.824	57.731	3.255	*	48.867	4.365	56.986	4.881	***
MCV	51.138	1.601	50.544	1.565		50.353	1.945	51.257	1.989	
MCH	15. 746	0.626	15.219	0.533	.	15.910	0.595	19.086	6.305	*
MCHC	30.754	0.818	30.113	0.868		31.617	0.841	25.386	7.625	***
RDW	29.638	2.684	28.469	1.199		27.667	1.490	30.986	4.080	**

3.4. The RBC picture of males under low and high food availability

During the potential period of ripe seeds, several erythrocyte parameters differed significantly among years of low and high food availability. RBC, Hb, Hct, and MCV were significantly higher in males under high food availability compared to males under food limitation, whereas the MCHC was significantly lower (Models: $food_{low}$ + ID random; n=64; DF=49; RBC^2: Estimate=22.43, p=0.017; Hb^2: Estimate=53.938, p<0.001; Hct^2: Estimate=731.204, p<0.001; Models sqrtMCV, 1/MCHC: see Tables A19-20, Fig. 2).

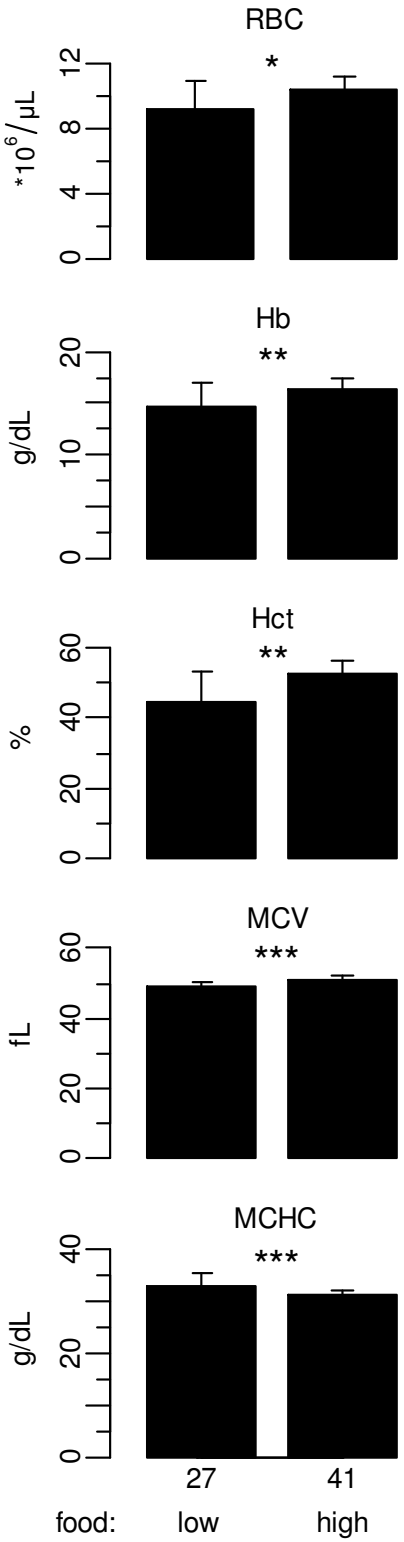

Fig. 2 RBC, Hb, Hct, MCV, and MCHC of male edible dormice of HE, SF, JH, and BG during the period of ripe seeds in the low vs. the high food/repro years (low: 2012 vs high: 2013 and 2014; mean + SD). Sample sizes are quoted below the bar labels.

3.5. Haematological study site differences

Males

The study site with the highest population density (BG, see Table 1), was set as reference for investigating study site differences. During PostH, Hb and Hct were higher in males of BG compared to HE and SF (tendency for Hb in HE, significance for Hb and Hct in SF). Furthermore, males from BG exhibited significantly higher values in MCV compared to HE, SF, and BS and the index MCH was higher in BG than in BS. In HE, a significantly higher MCHC was found compared to BG (Tables 4, A21-25). During Rep, a tendency to higher RBC counts as well as significantly higher Hb and Hct levels in SF were found. The MCV was significantly higher in males of HE, SF, and JH, compared to BG, but higher MCH values were found in SF (tendency) and JH (p=0.049) (Tables 5, A26-31).

Females

Upon termination of hibernation, RBC counts and Hb were significantly lower in females from BG compared to SF, JH, and BS, whereas females from HE showed comparable values in these parameters. In BS, the Hct was also significantly higher compared to BG. The MCV and the MCH in BG was significantly higher compared to HE and SF and the MCH was furthermore significantly lower in BS (Tables 6, A32-38).

Table 4. PostH haematological parameters of male edible dormice of the different study sites HE, SF, JH, BS, and BG in the reproductive years 2013 and 2014 (mean and SD). Study sites are arranged in ascending order according to their population densities from left to right. Reference for study site comparisons was BG. Significant differences according to the linear mixed models are marked by asterisks.

	HE (n=10)			SF (n=6)			JH (n=10)		BS (n=15)			BG (n=14)	
	mean	SD		mean	SD		mean	SD	mean	SD		mean	SD
RBC	10.43	1.36		10.39	0.56		11.28	0.70	11.16	0.80		10.94	0.94
Hb	15.79	1.38	.	15.55	1.23	*	17.35	1.09	16.45	1.06		16.86	1.45
Hct	49.89	6.77	*	46.87	11.38	*	56.90	4.17	54.75	3.75		55.51	5.27
MCV	47.80	1.23	***	49.08	1.68	*	50.45	1.07	49.09	1.62	**	50.72	1.46
MCH	15.26	1.28		14.95	0.45		15.38	0.31	14.75	0.55	*	15.43	0.44
MCHC	31.91	2.67	*	30.45	0.94		30.52	0.86	30.05	0.93		30.44	0.90
RDW	27.94	1.76		27.82	1.41		27.28	1.15	28.21	1.81		29.07	3.92

Table 5. Rep haematological parameters of male edible dormice of the different study sites HE, SF, JH, BS, and BG in the reproductive years 2013 and 2014 (mean and SD). Study sites are arranged in ascending order according to their population densities from left to right. Reference for study site comparisons was BG. Significant differences according to the linear mixed models are marked by asterisks.

	HE			SF			JH			BS			BG	
	n=15			n=12			n=10			n=13			n=10	
	mean	SD		mean	SD		mean	SD		mean	SD		mean	SD
RBC	9.14	0.96		10.49	1.07	•	9.99	1.22		10.32	0.85		10.16	1.01
Hb	14.33	1.47		16.48	1.11	*	15.87	1.72		15.82	1.15		15.19	1.81
Hct	48.42	4.96		55.78	4.88	**	52.66	6.66		53.18	4.76		51.75	4.25
MCV	53.02	1.82	**	53.26	1.47	**	52.74	0.95	*	51.55	1.18		51.08	1.96
MCH	15.69	0.75		15.78	0.79	•	15.92	0.44	*	15.35	0.45		14.95	0.94
MCHC	29.62	0.89		29.62	1.12		30.22	0.85		29.79	0.83		29.28	1.53
RDW	31.77	2.57		32.25	2.55		30.57	1.52		31.02	1.93		30.66	4.44

Table 6. PostH haematological parameters of female edible dormice of the different study sites HE, SF, JH, BS, and BG in the reproductive years 2013 and 2014 (mean and SD). Study sites are arranged in ascending order according to their population densities from left to right. Reference for study site comparisons was BG. Significant differences according to the linear mixed models are marked by asterisks.

	HE			SF			JH			BS			BG	
	n=13			n=7			n=12			n=12			n=14	
	mean	SD		mean	SD		mean	SD		mean	SD		mean	SD
RBC	9.86	0.57		11.19	0.49	**	10.56	1.01	*	11.07	1.09	**	9.76	0.89
Hb	15.02	1.14		16.99	0.86	*	16.63	1.53	*	16.80	1.19	***	15.39	1.28
Hct	48.32	3.56		55.17	2.65		53.25	5.47		55.47	4.55	*	47.66	8.75
MCV	48.98	1.32	*	49.31	1.44	**	50.42	2.08		50.23	2.04		50.14	1.52
MCH	15.22	0.52	*	15.19	0.52	*	15.77	0.53		15.24	0.74	*	15.79	0.46
MCHC	31.08	0.85		30.77	0.30		31.28	1.00		30.35	1.30		31.49	1.25
RDW	26.82	1.15	*	27.91	1.09		27.89	2.20		29.91	4.46		27.65	1.24

3.6. Incidence of Haemoglobinuria

During PostH and Rep of high reproductive years, dark urine, associated with haemoglobinuria, was detected more frequently in females than in males (Chi-squared test: X^2=13.75, df=1, p<0.001; n=319). In males, the prevalence ratio of dark urine was in reproductive as well as in the non-reproductive year lowest during PostH and increased until PreH (Fig. 3). However, this seasonal increase was only significant for PreH during the non-reproductive year (2012, Chi-squared-test: X^2=19.43, df=2, p<0.001, n=52; 2013/14, Chi-squared-test: X^2=4.99, df=2, p=0.082, n=126). Also in

females, the same seasonal pattern prevailed in the in the low food/repro year (Chi-squared-test: X^2=6.46, df=2, p=0.046, n=52). In high food/repro years, frequency of haemoglobinuria was higher during PostH and Rep than during PreH. However, this seasonal variation was statistically not significant (Chi-squared-test: X^2=1.31, df=2 , p=0.519, n=175 ; Fig. 3).

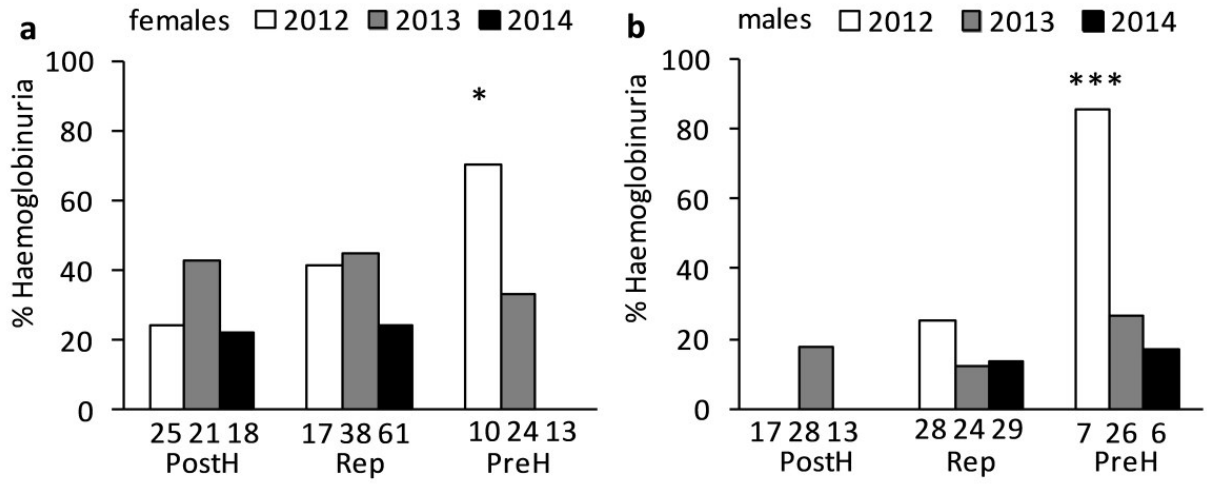

Fig. 3 Prevalence ratio (%) of haemoglobinuria in (a) female and (b) male edible dormice during PostH, Rep, and PreH of their active season in the low food/repro year 2012 and the high food/repro years 2013 and 2014. Sample sizes are quoted below the bar labels. Significant differences between the periods are indicated by asterisks, difference between the sexes are quoted in the result section.

4. Discussion

4.1. Seasonal variations in RBC indices

We found clear seasonal patterns in various RBC indices in both sexes. Compared to PostH, we detected a clear drop in RBC counts, Hct and Hb in both sexes during mid-summer, the period of high reproductive activity. These haematological alterations generally indicate an impairment in body condition and health status (Boonstra et al., 2001) and moreover, suggest the manifestation of an anaemia, especially in females of our study, where these declines were more pronounced than in males. Reductions in RBC counts and Hct have also been shown to be associated with reproductive activity in other species (Boonstra et al., 2001; Kalmbach et al., 2004), although these studies did not analyze the underlying mechanism. In our study, we observed the manifestation of a macrocytic anaemia during reproduction in both sexes, as the MCV was significantly increased. The most likely causes for macrocytosis in our wild dormouse population are either megalocytes or increased numbers of reticulocytes. Megalocytes occur under perturbations in DNA synthesis due to

deficiencies in vitamin B12 or folic acid and are huge RBCs with an exceptionally high content of haemoglobin (high MCH and MCHC), whereas the reticulocyte count elevates after substantial RBC loss when erythropoiesis is considerably accelerated as a regenerative response (Aslinia et al., 2006; Tyler and Cowell, 1996). The latter condition seems to prevail in males of our study as indicated by the typical increase in MCV and RDW (Tyler and Cowell, 1996), while the reductions in RBC counts, Hb, and Hct did not reflect a severe anaemia. Consequently, considerable RBC haemolysis and degradation associated with strong competitive exercise and erythrocyte senescence (Mairbäurl, 2013; Santos-Silva et al., 2001) occurred before and during mating and is not completely compensated by erythrocyte production, presumably due to a lack of high quality food. Gestation as well as lactation are often hallmarked by anaemia due to increased nutritional burden and high oestrogen levels that impair erythropoiesis (Blobel and Orkin, 1996; Dukes and Goldwasser, 1961; Kalmbach et al., 2004; Metz, 1970). These effects are often associated with iron or folic acid deficiency (Metz, 1970; Picciano, 2003; Suckow et al., 2005). However, anaemia during gestation can be relative (i.e. physiologic), due to an increased demand in total blood volume which is to a large extend achieved through dilution and consequently results in only slight decreases in Hct and Hb in peripheral blood (Scanlon et al., 2000; Tyler and Cowell, 1996). The strong reductions in RBC and Hb observed in females of our study rule out a relative anaemia. Instead, the subsidiary increases in MCV, MCH and MCHC point to a folate deficiency anaemia (Aslinia et al., 2006; Metz, 1970). An ancillary effect of folate deficiency anaemia is an accelerated haemolysis of the ineffective megaloblastic RBCs (Koury and Ponka, 2004), that accounts for only mild increases in MCV, as observed in our study, and furthermore intensifies the degradation of RBCs. The examination of urine colour revealed high frequencies of haemoglobinuria in females during Rep that indicate strong intravascular haemolysis, presumably caused by ineffective megalocytes but potentially also by further influences that can induce haemolysis (Keohane et al., 2015; Sivilotti, 2004). In both sexes, blood loss mediated by parasites (Weiss and Wardrop, 2011) can be excluded to have caused the haematological impairments during Rep because tick and endoparasite infestation rates were nearly absent during June and July and increased after the mating period (Fietz et al., 2015); Havenstein and Fietz, unpubl. data).

Edible dormice exhibit strongly elevated stress hormone levels during reproduction (Havenstein et al., unpublished data) leading to a characteristic physiological stress response in immune cell counts (Havenstein et al., 2016). Based on results of this study we further suggest that the high stress level during reproduction impacts the oxygen delivery system of male and female dormice in different ways: in males, GCs increase oxidative damage and RBC aging (Lutz and Bogdanova, 2013), but

simultaneously stimulate a regenerative response, i.e. the generation of erythrocytes (Lodish et al., 2010), preventing a severe RBC decay whereas in females the sex hormone status in association with folate deficiency hampers the positive effects of stress hormones and rather results in GC-induced oxidative damage and haemolysis (Keohane et al., 2015; Sivilotti, 2004). Summed up, in both sexes haematological changes observed during Rep apparently result from a superposition of different effects, namely nutrient deficiencies associated with increased demands as well as reproductive hormones, physical activity, stress and senescence of RBCs due to extended periods of low cellular activity during the antecedent hibernation period (see below).

In contrast to stress-induced alterations in the leukocyte differential that persisted until the end of the active season (Havenstein et al., 2016), the oxygen delivery system recovers largely after Rep, probably due to the availability of high quality food that enabled an accelerated erythropoiesis. Nevertheless, the recovery is incomplete, particularly in females that incur high reproductive investment through lactation until late in the active season.

4.2. Timing of RBC death and renewal

The frequently high prevalence of haemoglobinuria suggests that in edible dormice regularly occurs a strong RBC decay throughout their active season. This seems to be related primarily to the extended hibernation period resulting in accumulated senescent RBCs in spring and early summer. In the light of pre-hibernation haemoglobinuria we furthermore suggest that dormice have developed a preparatory mechanism that replaces erythrocytes with early signs of senescence (e.g. lipid peroxidation, membrane-bound haemoglobin; (Kumar and Rizvi, 2014; Lutz and Bogdanova, 2013) as senescent RBCs might become a source of reactive oxygen species (ROS) and potential damage in the course of hibernation (Belcher et al., 2009). This removal might be an important preparatory mechanism that mediates the noteworthy resilience in hibernators against ischaemia-reperfusion damage (Carey et al., 2000; Lindell et al., 2005; Zancanaro et al., 1999), especially in a non-reproductive year when dormice are in an energy saving mode (see below) which retards RBC aging (Mairbäurl, 2013; Santos-Silva et al., 2001) and presumably results in the accumulation of high proportions of senescent erythrocytes at the end of the active season. Conversely, during reproductive years a marked erythrocyte decay (decreasing RBC counts, considerable frequency of haemoglobinuria) and replacement occurs already during the antecedent reproductive period, resulting in a lower proportion of senescent RBCs during PreH that require renewal. This is supported by the higher MCV and MCH in both sexes during PreH that presumably indicate a younger pool of RBCs compared to PostH (Lutz and Bogdanova, 2013; Willekens et al., 1997). As lactation lasts until shortly before females start hibernating, they seem to be incapable of performing a preparative RBC

replacement before the onset of hibernation, as supported by declining frequencies of haemoglobinuria and the fact that females do not recover completely from their acquired anaemia. Accordingly, females potentially experience elevated damage through ROS during hibernation, which might be one determinant for the increased mortality during PostH following a reproductive year (Lebl et al., 2011; Ruf et al., 2006).

In hibernators, the effects of prolonged torpor bouts and arousals determine the individual's performance after emergence from hibernation (Havenstein et al., 2016) as cell processes, including haematopoiesis (Carey et al., 2003; Kruman, 1992; Lyman et al., 1957) as well as erythrocyte senescence are extremely reduced during torpor, but are reactivated during arousals. The retarded senescence explains why hibernators are not anaemic upon emergence (Brace, 1953; Brock, 1960; Lyman et al., 1957), however, large proportions of erythrocytes are expected to have fulfilled a predominant part of their life span at the end of hibernation (Brace, 1953; Brock, 1960; Kurata et al., 1993; Lyman et al., 1957; Szilagyi and Senturia, 1974). In our study, we apparently found an enhancement in oxygen carrying capacity in the course of the hibernation period as in both sexes RBC counts, Hb, and Hct were higher during $PostH_{short}$ compared to preceding $PreH_{short}$ levels. As creatinine levels in the urine of edible dormice were not elevated after hibernation was terminated (Havenstein et al., in press), dehydration seem not to be the cause for these changes. Furthermore, in females, the remaining RBC indices (MCV, MCH, MCHC, RDW) suggest a comparatively younger RBC pool whereas in males the slight decreases (non-significant) in MCV and MCH rather indicate a tendency to elder RBCs after hibernation (Lutz and Bogdanova, 2013; Willekens et al., 1997). The high prevalence ratio of haemoglobinuria (34-42 %) during PostH in females indicates a massive RBC destruction, which was not observed in males. Accordingly, besides the accumulation of senescent erythrocytes a moderate erythrocyte production seems to have occurred during euthermic arousals in the course of the hibernation period, especially in females. The difference between the sexes supports our notion that males are more capable to regenerate their RBC pool before hibernation and hence their RBC pool endures longer as demonstrated by a less pronounced RBC destruction during PostH than females. However, the oxygen carrying capacity of males during mating was distinctly more impaired (lower RBC, Hb, and Hct values) when they had already reproduced in the preceding year. Hence, in both sexes, we detected signs of physiological impairments for prolonged times after the reproductive period (see also (Boonstra, 2005; Kalmbach et al., 2004), meaning that individuals trade off health and body condition for reproductive success (Santos-Silva et al., 2001). This is in line with previous findings of immune cell alterations and reduced survival associated with

reproduction (Havenstein et al., 2016; Lebl et al., 2011; Ruf et al., 2006) but so far physiological impairments were not shown to last until the subsequent active season.

4.3. Food availability

For specifically evaluating the effects of food availability on the oxygen delivery system, we assessed erythrocyte parameters in adult males under high and low food availability. As expected, males under food limitation exhibited significantly lower RBC counts, Hb, and Hct, which is often associated with a low nutritional status (Beldomenico et al., 2008) and is normally interpreted as a poor health status and a compromised oxygen delivery to body tissues (Huitu et al., 2007; Kalmbach et al., 2004; Potti et al., 1999; Weinsier et al., 1979). Ecophysiological studies clearly showed that edible dormice remain in an energy saving mode during extended periods of food limitation, with a greatly reduced oxygen consumption (Bieber and Ruf, 2009); Langer et al., unpublished data). Therefore, we suggest that the low oxygen delivery capacity result from a reduced erythropoiesis sufficient to meet the physiological needs during a year of low food availability and activity instead of representing an impairment in body condition. This is supported by slightly higher MCV and lower MCHC values in males under high food availability indicating a somewhat younger RBC pool and larger RBC production (Lutz and Bogdanova, 2013; Willekens et al., 1997). As survival rates in dormice were even higher in low food than in high food years (Lebl et al., 2011; Ruf et al., 2006), the reduced erythropoiesis presumably represents part of the energy saving strategy of this small hibernator during prolonged periods of low food availability. Relying on the general assumption, that a high Hct or Hb reflect a better health and body condition (Boonstra et al., 2001; Franzmann and Leresche, 1978; Jégo et al., 2014, p.; Johnstone et al., 2015; Ots et al., 1998) would be misleading here. Consequently, it is important to consider further physiological and fitness parameters to elucidate mechanisms underlying changes of physiological states.

4.4. Study site effects

Study site differences were most pronounced at the beginning of the active season. Referring to RBC counts, Hb, and Hct, females from SF, JH, and BS emerge from hibernation with a better oxygen delivery capacity compared to BG and HE, the study sites with the highest and the lowest population density. In conjunction with slight differences in MCV and MCH, we assume that at the end of hibernation and upon emergence the strength of erythrocyte degradation and production, i.e. investment into erythropoiesis, varies among the study sites.

In males of the study site BG (highest population density) Hb and Hct values during PostH were higher than those of the study sites with the lowest population densities (HE and SF) and further parameters indicate a slightly younger RBC pool in BG. However, during Rep, the oxygen delivery

capacity of dormice in all sites (except SF) ranges on comparable levels but higher values in MCV and MCH suggest a stronger investment into RBC production in the study sites with the lowest population densities (HE, SF, JH) (Aslinia et al., 2006; Tyler and Cowell, 1996). Thus, RBC loss was also strongest in the study sites HE and JH, rendering this stronger regenerative response necessary. Accordingly, as in females, the strength of erythrocyte destruction and renewal may vary among sites, which, however, in neither sex can be explained by varying population densities. In our previous studies we found that individuals in BG are larger and heavier (Fietz and Weis-Dootz, 2012) and experience least physiological stress (Havenstein et al., 2016). The lower regenerative response in BG during Rep is in line with the lower stress and activity levels. Therefore, we assume that males from BG face favorable environmental conditions (e.g. predation pressure) and are able to allocate their energy predominantly into reproductive activities (Moore and Hopkins, 2009) whereas males in other study sites need to mount a stronger regenerative response. Such varied site-specific characteristics may ultimately determine differences in performance and trade-offs. Our analyses again demonstrate that focussing only on single parameters during short time periods (Boonstra et al., 2001; Franzmann and Leresche, 1978; Jégo et al., 2014, p.; Johnstone et al., 2015; Ots et al., 1998) might be misleading when assessing the condition of individuals, as this ignores potential differences in the strength of erythropoiesis and associated costs.

5. Conclusions

Edible dormice experience severe impairments in their oxygen delivery system during reproduction. The high demands of reproduction, including hormonal changes and stress, plus the lack of high quality food cause a strong erythrocyte decay in conjunction with a regenerative response in males and a folic acid deficiency anaemia in females. After periods of high reproductive activity, the impairment in the oxygen delivery system can persist throughout the following year and cause even stronger erythrocyte deterioration upon subsequent reproduction. No immediate effect on the oxygen delivery system could be found through hibernation, however, an accumulation of senescent erythrocytes seems to occur after hibernation. In general, the activity level of the individual seems to determine the pace of erythrocyte senescence. High prevalence of haemoglobinuria shortly before hibernation suggests that the RBC pool is renewed, which may be a preparative mechanism to reduce oxidative stress during hibernation, preventing ischaemia-reperfusion damage. The strength of erythrocyte destruction and renewal seems to vary among sites and may ultimately determine site-specific fitness trade-offs. Our study reveals that in both sexes the oxygen delivery system responds sensitively to a variety of effects that may superimpose. Therefore, a comprehensive haemogram represents a powerful tool for assessing the current physiological status of an individual

and investigating impacts, allocation decisions and mechanisms underlying physiological performance and fitness parameters in wild populations.

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Abbreviations: *GCs* glucocorticoids - *PostH* post-hibernation period - *Rep* reproductive period - *PreH* pre-hibernation period - $PostH_{short}$ shortened post-hibernation period - $PreH_{short}$ shortened pre-hibernation period - *RAL* Reichsausschuss für Lieferbedingungen, a colour matching system with scaled colours - *RBC* red blood cell - T_b body temperature

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Appendix Chapter III

Table A1. Estimates of fixed effects of the general linear mixed model for the RBC counts of male edible dormice collected in all study sites during the entire active period of 2013 and 2014. Model: RBC^2 = period + year+ ID random, n=163

RBC^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		3.922	1	0.048	14714.661	7429.858	101	1.980	0.050
Period PostH		25.038	2	<0.001					
	Rep				-18.391	3.688	58	-4.987	<0.001
	PreH				-11.565	4.294	58	-2.694	0.009
Year		3.859	1	0.049	-7.250	3.690	58	-1.964	0.054

Table A2. Estimates of fixed effects of the general linear mixed model for the Hb of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: Hb^2 = period + Body condition + ID random, n=163

Hb^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		72.229	1	<0.001	216.917	25.523	101	8.499	<0.001
Period PostH		11.767	2	0.003					
	Rep				-25.924	7.849	58	-3.303	0.002
	PreH				-18.487	9.553	58	-1.935	0.058
Body condition		5.137	1	0.023	21.393	9.439	58	2.266	0.027

Table A3. Estimates of fixed effects of the general linear mixed model for the Hct of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: Hct^2 = period + body condition + ID random, n=163

Hct^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		42.184	1	<0.001	2156.511	332.032	101	6.495	<0.001
Period PostH		3.938	2	0.140					
	Rep				-69.374	103.564	58	-0.670	0.506
	PreH				-244.807	123.383	58	-1.984	0.052
Body condition		5.231	1	0.022	280.425	122.612	58	2.287	0.026

Table A4. Estimates of fixed effects of the general linear mixed model for the MCV of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: lnMCV = period + year+ ID random, n=163

lnMCV		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		0.876	1	0.349	-5.151	5.503	101	-0.936	0.351
Period PostH		79.059	2	<0.001					
	Rep				0.024	0.003	58	8.750	<0.001
	PreH				0.009	0.003	58	2.782	0.007
Year		1.548	1	0.213	0.003	0.003	58	1.244	0.218

Table A5. Estimates of fixed effects of the general linear mixed model for the MCH of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: sqrt MCH = period + site + year+ ID random, n=163

sqrt MCH		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		8.914	1	0.003	-726.925	243.469	97	-2.986	0.004
Period PostH		12.324	2	0.002					
	Rep				0.344	0.121	58	2.839	0.006
	PreH				0.451	0.143	58	3.150	0.003
Study site		12.233	4	0.016					
	HE				0.162	0.200	97	0.810	0.420
	SF				0.066	0.204	97	0.325	0.746
	JH				0.193	0.200	97	0.967	0.336
	BS				-0.404	0.194	97	-2.089	0.039
Year		9.292	1	0.002	0.369	0.121	58	3.048	0.004

Table A6. Estimates of fixed effects of the general linear mixed model for the MCHC of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: $1/MCHC^2$ = period + year+ ID random, n=163

$1/MCHC^2$		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		6.685	1	0.010	0.076	0.030	101	2.586	0.011
Period PostH		38.502	2	<0.001					
	Rep				6.3E-05	1.5E-05	58	4.242	<0.001
	PreH				-3.2E-05	1.7E-05	58	-1.931	0.058
Year		6.499	1	0.011	-3.7E-05	1.5E-05	58	-2.549	0.014

Table A7. Estimates of fixed effects of the general linear mixed model for the RDW-SD of male edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: $1/RDW^2$ = period + ID random, n=163

$1/RDW^2$		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		2541.863	1	<0.001	1.3E-03	2.5E-05	101	50.417	<0.001
Period PostH		52.807	2	<0.001					
	Rep				-2.4E-04	3.4E-05	59	-6.935	<0.001
	PreH				-5.5E-05	3.7E-05	59	-1.469	0.147

Table A8. Estimates of fixed effects of the general linear mixed model for the RBC of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: RBC^2 ~ period + study site + ID random, n=175

RBC^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		548.555	1	<0.001	104.421	4.458	99	23.421	<0.001
Period PostH		63.538	2	<0.001					
	Rep				-31.284	3.926	69	-7.968	<0.001
	PreH				-15.015	3.980	69	-3.772	<0.001
Study site		16.605	4	0.002					
	HE				-5.177	5.639	99	-0.918	0.361
	SF				11.965	5.717	99	2.093	0.039
	JH				6.939	5.538	99	1.253	0.213
	BS				12.076	5.589	99	2.161	0.033

Table A9. Estimates of fixed effects of the general linear mixed model for the Hb of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: Hb^2 ~ period + study site + ID random

Hb^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		654.881	1	<0.001	262.783	10.269	99	25.591	<0.001
Period PostH		19.368	2	<0.001					
	Rep				-38.662	8.862	69	-4.363	<0.001
	PreH				-14.191	9.720	69	-1.460	0.149
Study site		16.539	4	0.002					
	HE				-41.038	12.780	99	-3.211	0.002
	SF				3.845	13.325	99	0.289	0.774
	JH				-5.019	12.857	99	-0.390	0.697
	BS				-12.323	12.604	99	-0.978	0.331

Table A10. Estimates of fixed effects of the general linear mixed model for the Hct of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: Hct sq = period + study site + ID random, n= 175

Hct		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		499.670	1	<0.001	2686.328	120.176	99	22.353	<0.001
Period PostH		20.425	2	<0.001					
	Rep				-468.508	103.716	69	-4.517	<0.001
	PreH				-247.010	113.812	69	-2.170	0.033
Study site		13.765	4	0.008					
	HE				-400.913	149.539	99	-2.681	0.009
	SF				90.709	155.926	99	0.582	0.562
	JH				-32.046	150.449	99	-0.213	0.832
	BS				-18.923	147.478	99	-0.128	0.898

Table A11. Estimates of fixed effects of the general linear mixed model for the MCV of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: sqrt MCV = period + study site + body condition + year + ID random, n= 175

sqrt MCV		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		7.409	1	0.006	-145.751	53.548	99	-2.722	0.008
Period PostH		22.082	2	<0.001					
	Rep				0.085	0.028	67	3.070	0.003
	PreH				0.132	0.029	67	4.541	<0.001
Study site		24.405	4	<0.001					
	HE				-0.136	0.036	99	-3.813	<0.001
	SF				-0.115	0.036	99	-3.185	0.002
	JH				-0.101	0.035	99	-2.902	0.005
	BS				-0.165	0.035	99	-4.787	<0.001
Body condition		11.075	1	0.001	-0.087	0.026	67	-3.328	0.001
Year		8.178	1	0.004	0.076	0.027	67	2.860	0.006

Table A12. Estimates of fixed effects of the general linear mixed model for the MCH of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: 1/MCH = period + study site + ID random, n= 175

1/MCH		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		11897.725	1	<0.001	0.063	0.001	99	109.077	<0.001
Period PostH		28.318	2	<0.001					
	Rep				-0.003	0.000	69	-5.167	<0.001
	PreH				-0.002	0.000	69	-3.864	<0.001
Study site		18.052	4	0.001					
	HE				0.001	0.001	99	1.847	0.068
	SF				0.001	0.001	99	1.756	0.082
	JH				0.001	0.001	99	1.073	0.286
	BS				0.003	0.001	99	4.021	<0.001

Table A13. Estimates of fixed effects of the general linear mixed model for the MCHC of female edible dormice collected in all study sites during the entire active periods of 2013 and 2014. Model: MCHC = period + ID random, n= 175

MCHC		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		53564.790	1	<0.001	31.039	0.134	103	231.441	<0.001
Period PostH		12.830	2	0.002					
	Rep				0.601	0.182	69	3.296	0.002
	PreH				0.553	0.190	69	2.908	0.005

Table A14. Estimates of fixed effects of the general linear mixed model for the RBC count of male edible dormice collected in all study sites during $PreH_{short}$ 2013 and $PostH_{short}$ 2014. Model: RBC^2 = period $PreH_{short}/PostH_{short}$ + study site + ID random, n= 40

RBC		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		261.502	1	<0.001	100.616	6.222	31	16.171	<0.001
$PreH_{short}$		17.700	1	<0.001					
	$PostH_{short}$				23.688	5.630	3	4.207	0.025
Study site		8.457	4	0.076					
	HE				19.002	8.534	31	2.226	0.033
	SF				5.808	10.613	31	0.547	0.588
	JH				18.068	8.142	31	2.219	0.034
	BS				2.591	8.421	31	0.308	0.760

Table A15. Estimates of fixed effects of the general linear mixed model for the Hb of male edible dormice collected in all study sites during $PreH_{short}$ 2013 and $PostH_{short}$ 2014. Model: 1/Hb = period $PreH_{short}/PostH_{short}$ + study site + ID random, n= 40

1/Hb		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		2579.285	1	<0.001	0.062	0.001	31	50.787	<0.001
$PreH_{short}$		11.931	1	0.001					
	$PostH_{short}$				-0.004	0.001	3	-3.454	0.041
Study site		10.338	4	0.035					
	HE				-0.003	0.002	31	-1.492	0.146
	SF				0.000	0.002	31	-0.212	0.834
	JH				-0.003	0.002	31	-1.930	0.063
	BS				0.002	0.002	31	1.095	0.282

Table A16. Estimates of fixed effects of the general linear mixed model for the Hct of male edible dormice collected in all study sites during $PreH_{short}$ 2013 and $PostH_{short}$ 2014. Model: logHct = period $PreH_{short}/PostH_{short}$ + ID random, n= 40

logHct		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		91408.030	1	<0.001	1.726	0.006	35	302.338	<0.001
$PreH_{short}$		21.786	1	<0.001					
	$PostH_{short}$				0.037	0.008	3	4.668	0.019

Table A17. Estimates of fixed effects of the general linear mixed model for the MCH of male edible dormice collected in all study sites during $PreH_{short}$ 2013 and $PostH_{short}$ 2014. Model: 1/MCH = period $PreH_{short}$/$PostH_{short}$ + ID random, n= 40

1/MCH	Chisq	Df	p	Estimate	SE	Df	t	p
Intercept	19119.417	1	<0.001	0.064	4.6E-04	35	138.273	<0.001
$PreH_{short}$	6.563	1	0.010					
$PostH_{short}$				0.002	6.1E-04	3	2.562	0.083

Table A18. Estimates of fixed effects of the general linear mixed model for the MCHC of male edible dormice collected in all study sites during $PreH_{short}$ 2013 and $PostH_{short}$ 2014. Model: 1/MCHC = period $PreH_{short}$/$PostH_{short}$ + ID random, n= 40

1/MCHC	Chisq	Df	p	Estimate	SE	Df	t	p
Intercept	31129.04	1	<0.001	0.033	1.8E-04	35	176.434	<0.001
$PreH_{short}$	4.651	1	0.031					
$PostH_{short}$				6.2E-04	2.9E-04	3	2.156	0.120

Table A19. Estimates of fixed effects of the general linear mixed model for the MCV of males collected in He, SF, JH, and BG during the period of ripe seeds (August + September) in the low food year 2012 and the high food years 2013 and 2014. Model: sqrt MCV = $food_{low}$ + study site + ID random, n= 64

sqrt MCV		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		26955.679	1	<0.001	6.925	0.042	46	164.182	<0.001
Food	low	29.651	1	<0.001					
	high				0.130	0.024	46	5.445	<0.001
Study site		10.701	3	0.013					
	HE				-0.072	0.031	46	-2.327	0.024
	SF				-0.094	0.032	46	-2.948	0.005
	JH				-0.077	0.034	46	-2.288	0.027

Table A20. Estimates of fixed effects of the general linear mixed model for the MCHC of males collected in He, SF, JH, and BG during the period of ripe seeds (August + September) in the low food year 2012 and the high food years 2013 and 2014. Model: 1/MCHC = $food_{low}$ + study site + ID random, n= 64

1/MCHC		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		571.056	1	<0.001	8.8E-04	3.7E-05	46	23.897	<0.001
Food	low	16.879	1	<0.001					
	high				8.6E-05	2.1E-05	46	4.108	<0.001
Study site		13.911	3	0.003					
	HE				-8.2E-05	2.7E-05	46	-3.058	0.004
	SF				1.5E-05	2.8E-05	46	0.525	0.602
	JH				-2.6E-05	2.9E-05	46	-0.886	0.380

Table A21. Estimates of fixed effects of the general linear mixed model for the Hb of male edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: Hb sq = study site + ID random, n = 55

Hb		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		584.035	1	<0.001	285.468	11.812	36	24.167	<0.001
Study site BG		10.943	4	0.027					
	HEHL				-34.758	19.226	36	-1.808	0.079
	SF				-44.232	21.321	36	-2.075	0.045
	JH				15.778	17.900	36	0.881	0.384
	BS				-15.799	16.781	36	-0.941	0.353

Table A22. Estimates of fixed effects of the general linear mixed model for the Hct of male edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: Hct^2 = study site + ID random, n = 55

Hct^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		302.403	1	<0.001	3107.704	178.709	36	17.390	<0.001
Study site		14.087	4	0.007					
	HEHL				-663.590	295.844	36	-2.243	0.031
	SF				-864.080	321.132	36	-2.691	0.011
	JH				124.395	268.579	36	0.463	0.646
	BS				-125.164	256.307	36	-0.488	0.628

Table A23. Estimates of fixed effects of the general linear mixed model for the MCV of male edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: ln MCV = study site + ID random, n = 55

ln MCV		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		208375.532	1	<0.001	1.705	0.004	36	456.482	<0.001
Study site		23.996	4	<0.001					
	HEHL				-0.026	0.006	36	-4.306	<0.001
	SF				-0.014	0.007	36	-2.065	0.046
	JH				-0.003	0.006	36	-0.475	0.638
	BS				-0.015	0.005	36	-2.892	0.007

Table A24. Estimates of fixed effects of the general linear mixed model for the MCH of male edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: ln MCH = study site + ID random, n = 55

ln MCH		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		41289.795	1	<0.001	1.187	0.006	36	203.199	<0.001
Study site BG		9.824	4	0.044					
	HEHL				0.003	0.010	36	0.352	0.727
	SF				-0.014	0.010	36	-1.323	0.194
	JH				-0.001	0.009	36	-0.064	0.949
	BS				-0.021	0.008	36	-2.444	0.020

Table A25. Estimates of fixed effects of the general linear mixed model for the MCHC of male edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: 1/MCHC = study site + ID random, n = 55

1/MCHC		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		6825.351	1	<0.001	0.033	0.000	36	82.616	<0.001
Study site BG		13.629	4	0.009					
	HEHL				-0.002	0.001	36	-2.970	0.005
	SF				0.000	0.001	36	0.028	0.978
	JH				0.000	0.001	36	-0.304	0.763
	BS				0.000	0.001	36	0.654	0.517

Table A26. Estimates of fixed effects of the general linear mixed model for the RBC counts of male edible dormice collected in all study sites during Rep in the reproductive years 2013 and 2014. Model: RBC^2= study site + year + ID random, n = 60

RBC^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		352.013	1	<0.001	104.604	5.575	41	18.762	<0.001
Study site		14.206	4	0.007					
	HE				-10.091	7.601	41	-1.328	0.192
	SF				14.074	7.757	41	1.814	0.077
	JH				3.570	8.046	41	0.444	0.660
	BS				8.504	7.536	41	1.128	0.266
Year		20.539	1	<0.001	-23.979	5.291	13	-4.532	<0.001

Table A27. Estimates of fixed effects of the general linear mixed model for the Hb of male edible dormice collected in all study sites during Rep in the reproductive years 2013 and 2014. Model: Hb^2= study site + year + ID random, n = 60

Hb^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		293.269	1	<0.001	238.146	13.906	41	17.125	<0.001
Study site		13.968	4	0.007					
	HE				-8.449	19.219	41	-0.440	0.663
	SF				49.746	19.313	41	2.576	0.014
	JH				28.408	20.047	41	1.417	0.164
	BS				26.635	18.840	41	1.414	0.165
Year		13.464	1	<0.001	-46.423	12.652	13	-3.669	0.003

Table A28. Estimates of fixed effects of the general linear mixed model for the Hct of male edible dormice collected in all study sites during Rep in the reproductive years 2013 and 2014. Model: Hct^2= study site + year + ID random, n = 60

Hct^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		341.419	1	<0.001	2719.069	147.155	41	18.478	<0.001
Study site		16.749	4	0.002					
	HE				-33.173	202.084	41	-0.164	0.870
	SF				636.633	204.600	41	3.112	0.003
	JH				298.060	212.289	41	1.404	0.168
	BS				315.403	199.170	41	1.584	0.121
Year		26.898	1	<0.001	-711.866	137.259	13	-5.186	<0.001

Table A29. Estimates of fixed effects of the general linear mixed model for the MCV of male edible dormice collected in all study sites during Rep in the reproductive years 2013 and 2014. Model: ln MCV= study site + ID random, n = 60

ln MCV		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		180660.17	1	<0.001	1.708	0.004	41	425.041	<0.001
Study site		18.314	4	0.001					
	HE				0.016	0.005	41	3.131	0.003
	SF				0.018	0.005	41	3.359	0.002
	JH				0.014	0.006	41	2.484	0.017
	BS				0.004	0.005	41	0.785	0.437

Table A30. Estimates of fixed effects of the general linear mixed model for the MCH of male edible dormice collected in all study sites during Rep in the reproductive years 2013 and 2014. Model: MCH^2= study site + year + ID random, n = 60

MCH^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		1271.571	1	<0.001	226.049	6.339	41	35.659	<0.001
Study site		6.423	4	0.170					
	HE				12.091	8.786	41	1.376	0.176
	SF				17.034	8.794	41	1.937	0.060
	JH				18.523	9.133	41	2.028	0.049
	BS				4.690	8.591	41	0.546	0.588
Year		17.434	1	<0.001	23.581	5.648	13	4.175	0.001

Table A31. Estimates of fixed effects of the general linear mixed model for the MCHC of male edible dormice collected in all study sites during Rep in the reproductive years 2013 and 2014. Model: $MCHC^2$ = year + ID random, n= 60

$MCHC^2$	Chisq	Df	p	Estimate	SE	Df	t	p
Intercept	12141	1	<0.001	862.483	7.827504	45	110.1862	<0.001
Year	38.608	1	<0.001	82.5792	13.29013	13	6.21358	<0.001

Table A32. Estimates of fixed effects of the general linear mixed model for the RBC of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: RBC= study site + ID random, n = 58

RBC		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		1753.968	1	<0.001	9.763	0.233	38	41.880	<0.001
Study site BG		25.613	4	<0.001					
	HE				0.097	0.336	38	0.288	0.775
	SF				1.428	0.404	38	3.537	0.001
	JH				0.799	0.343	38	2.329	0.025
	BS				1.303	0.343	38	3.797	<0.001

Table A33. Estimates of fixed effects of the general linear mixed model for the Hb of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: Hb^2= study site + year + ID random, n = 58

Hb^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		472.616	1	<0.001	238.159	10.955	38	21.740	<0.001
Study site BG		22.012	4	<0.001					
	HE				-10.898	15.814	38	-0.689	0.495
	SF				50.453	18.841	38	2.678	0.011
	JH				39.768	16.111	38	2.468	0.018
	BS				45.686	16.172	38	2.825	0.008

Table A34. Estimates of fixed effects of the general linear mixed model for the Hct of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: Hct^2= study site + year + ID random, n = 58

Hct^2		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		309.227	1	<0.001	2342.260	133.198	37	17.585	<0.001
Study site BG		8.907	4	0.063					
	HE				-127.047	199.156	37	-0.638	0.528
	SF				282.331	288.031	37	0.980	0.333
	JH				272.663	220.361	37	1.237	0.224
	BS				469.747	227.281	37	2.067	0.046
Year		6.083	1	0.014	425.315	172.445	37	2.466	0.018

Table A35. Estimates of fixed effects of the general linear mixed model for the MCV of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: lnMCV = study site + year + ID random, n = 58

lnMCV		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		192170.4	1	<0.001	1.700	0.004	37	438.373	<0.001
Study site BG		11.411	4	0.022					
	HE				-0.015	0.006	37	-2.556	0.015
	SF				-0.025	0.008	37	-2.959	0.005
	JH				-0.008	0.006	37	-1.319	0.195
	BS				-0.010	0.007	37	-1.538	0.133
Year		10.623	1	0.001	0.016	0.005	37	3.259	0.002

Table A36. Estimates of fixed effects of the general linear mixed model for the MCH of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: lnMCH = study site + ID random, n = 58

logMCH		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		67425.7	1	<0.001	1.199	0.005	38	259.665	<0.001
Study site BG		11.501	4	0.021					
	HE				-0.015	0.007	38	-2.250	0.030
	SF				-0.018	0.008	38	-2.316	0.026
	JH				-0.002	0.007	38	-0.316	0.754
	BS				-0.017	0.007	38	-2.404	0.021

Table A37. Estimates of fixed effects of the general linear mixed model for the MCHC of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: 1/MCHC = year + body condition + ID random, n = 58

1/MCHC	Chisq	f	p	Estimate	SE	Df	t	p
Intercept	1528.3	1	<0.001	0.035	0.001	41	39.094	<0.001
Year	6.994	1	0.008	0.001	0.000	41	2.645	0.012
Body condition	13.539	1	<0.001	-0.001	0.000	14	-3.680	0.003

Table A38. Estimates of fixed effects of the general linear mixed model for the RDW of female edible dormice collected in all study sites during PostH in the reproductive years 2013 and 2014. Model: 1/RDW = study site + year + body condition + ID random, n = 58

1/RDW		Chisq	Df	p	Estimate	SE	Df	t	p
Intercept		101.27	1	<0.001	0.031	0.003	37	10.063	<0.001
Study site BG		10.014	4	0.040					
	HE				0.003	0.001	37	2.321	0.026
	SF				0.002	0.002	37	1.580	0.123
	JH				0.002	0.001	37	1.410	0.167
	BS				0.000	0.001	37	0.078	0.938
Year		7.292	1	0.007	-0.003	0.001	37	-2.700	0.010
Body condition		3.396	1	0.065	0.002	0.001	14	1.843	0.087

Chapter IV: Stress hormones

Building the bridge between environment, physiology and life history: stress hormones in a small mammalian hibernator

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Abstract

Glucocorticoids (GCs) belong to the major players in the hormonal regulation of the body, exerting their far-reaching effects on numerous processes like energy allocation, immunity and behavior. Whereas moderate GC elevations serve as an adaption enabling individuals to cope with challenging situations, chronically increased levels are capable to impair other functions. Information on GC variation can therefore contribute to our understanding on the evolution of life history strategies and mechanisms underlying population declines. To examine variations in GC levels in wild edible dormice (*Glis glis*), we measured urinary cortisol levels and linked them to haematological parameters and survival. Dormice were sampled during their active season in five different study sites located in south-western Germany. During reproduction, cortisol levels showed an increase that amplified in consecutive reproductive periods demonstrating that reproduction is stressful for both sexes and can result in the accumulation of allostatic load. Coherence with previously revealed impairments in the immune and the oxygen delivery system and reduced survival emphasize the functional relevance of this increase. Before hibernation, elevated cortisol levels might play a role for entering hibernation. After emergence, moderate elevations in cortisol levels are obviously required to liberate energy from body stores to restore regressed organs. A negative energy status together with a deprived innate immune system likely contribute to increased mortality rates after emergence. Irregularly occurring prolonged food limitation did not result in elevated GCs. This highlights that food availability is predictable for edible dormice and that they are able to adjust energy expenditure without the need to extensively mobilize fuel from internal energy stores.

High population densities do not seem to entail adverse effects as individuals from the high density site even showed lowest cortisol levels and weakest haematological impairments. Investigating cortisol levels for a prolonged time and linking them to additional physiological parameters enabled us to disentangle the impacts of different stressors and elucidate physiological processes underlying different life history states and environmental conditions. Summarized, cortisol concentrations precisely reflect adjusted hormonal set points to challenging situations that help to reveal physiological and fitness trade-offs.

1. Introduction

The performance of wild animals is impacted by diverse, co-occurring challenges, which might ultimately affect their reproductive success and survival. Variations of physiological parameters in response to these challenges may be the key to assess their relative importance and to elucidate mechanisms that contribute to variations of fitness parameters (Cooke et al., 2013; Wikelski and Cooke, 2006). The hypothalamic-pituitary-adrenal (HPA) axis plays an important role in the regulation of nearly all vital body functions via releasing glucocorticoid steroid hormones (GCs). Besides affecting energy allocation, such as enhancing circulating glucose levels under physically demanding situations like aggressive encounters or periods of food scarcity, GCs act on different tissues and interact with the immune system and other components of the endocrine system, influencing diverse physiological and behavioral functions, such as fertility and reproduction, metabolism, and immune function (Landys et al., 2006; McEwen and Wingfield, 2003; Sapolsky et al., 2000). Corresponding to the concept of allostasis and the reactive scope model, elevated GC levels typically result from a changed allostatic load during any challenging event and an accordingly adjusted set point of GCs (and other mediators), thus it reflects physiological adaptions to maintain homeostasis (McEwen and Wingfield, 2003; Romero et al., 2009). Whereas short term elevations of GC levels are therefore crucial for survival, chronically increased GCs are capable to impair other body functions and ultimately hamper survival and reproduction (Sapolsky et al., 2000; Webster Marketon and Glaser, 2008).

Persistent stress and high GC levels interfere with reproductive success, by suppressing gonadal activity which reduces sex hormone production, induces a shortening of the luteal phase, thereby preventing fertilization, implantation and maintenance of early pregnancy (Kirby et al., 2009; Nakamura et al., 2008). Although increasing GC levels in the course of gestation are a widespread phenomenon in female mammals (Altemus et al., 1995; Atkinson and Waddell, 1995; Reeder et al., 2004), a strong GC increase can lead to severe complications, such as abortions and impairments in

upbringing the offspring (Cyr and Michael Romero, 2007; Ellenberg et al., 2007; Müllner et al., 2004; Sanderson et al., 2015)

Chronic stress is known to negatively impact health through an increased susceptibility to infections and decreased immune defense upon infections, reactivation of latent viruses, slow wound healing and the development of cancer. The underlying cause is an impairment in immune functions through chronically increased GC levels, including reductions in NK cell activity, lymphocyte proliferation, and antibody generation (Altemus et al., 2001; Dhabhar, 2009; Hamilton, 1974; Millán et al., 1996; Silberman et al., 2003; Webster Marketon and Glaser, 2008). Concerning circulating immune cell counts, high GC concentrations induce a rise the numbers of neutrophils and monocytes, while decreasing lymphocyte counts, the resulting increment in the P/L (phagocyte counts:lymphocyte counts) ratio represents a marker for stress. As elevations in the P/L ratio are caused in parts by GC-induced lymphocyte death and impaired neutrophil endothelial cell adhesion and diapedisis, which consequently impedes the migration of neutrophils to sites of inflammation (O'connor et al., 2000; Shi et al., 2003; Wang et al., 2002) these immune cell alterations most likely hallmark the beginning of the described deterioration of immune defenses.

Besides the immune system, the transport of oxygen by the highly specialized red blood cells (RBCs) represents another vital body function that responds sensitively to high CORT levels. Increased GCs exacerbate oxidative stress and reduce the protective antioxidant systems, resulting in a shortened life span of RBCs (Fibach and Rachmilewitz, 2008; Oishi et al., 1999; Orzechowski et al., 2002). In addition, high stress hormone levels can simultaneously boost the release of erythrocytes from the bone marrow into the blood by increasing the formation of colony-forming unit-erythroids by more than tenfold (Lodish et al., 2010). As GCs act on multiple body functions, information on GC level variations in regard to challenging situations and their impact on physiological parameters can contribute to our understanding on the evolution of life history strategies and on changes in performance and fitness of organisms.

Our study species, the edible dormouse (*Glis glis*) is a small arboreal rodent characterized by an extraordinarily long hibernation period of up to 8 months from the end of September until the end of May (Bieber et al., 2014). During hibernation, heart and metabolic rates are drastically diminished and body temperature is reduced to ambient temperature (Bieber et al., 2014; Ruf and Geiser, 2015; Wilz and Heldmaier, 2000). Caused by the irregular seed production of their main feeding tree species, the European beech (*Fagus sylvatica*), this small rodent is confronted with extended periods of limited food availability that may last longer than 1.5 years, resulting in high reproductive activity in full mast years and whole populations that remain sexually quiescent in years of mast failure (Lebl

et al., 2011; Ruf et al., 2006). As individuals within one population are highly synchronized in their yearly cycle (Fietz et al., 2009), however populations show strong local variations concerning body masses and population densities (Fietz & Weis-Dootz 2012). Accordingly, this system provides natural experimental conditions in which trade-offs should become apparent even on a population level. Previous examinations have shown that edible dormice are immunologically impaired when they emerge from hibernation. During reproduction, they show marked increases in the ratio of the P/L ratio, which represents a stress response of the immune system, as well as impairments in their oxygen delivery system. These changes are associated with reduced survival probabilities (Havenstein et al., 2016; Lebl et al. 2011; Ruf et al., 2006).

The aim of this study was to examine changes in the stress hormone level in dependency of influencing factors such as reproductive activity, food limitation, hibernation and high population density and link the results to previous findings on WBC and RBC impairments and survival. We therefore collected urine in free ranging edible dormice during their active season from May throughout mid-September 2012 -2014 in five different study sites in South-Western Germany and determined the concentration of baseline urinary cortisol corrected by creatinine (CORT).

2. Material and Methods

2.1. Study animal & study sites

The arboreal edible dormouse (*G. glis*) is the largest European dormouse with a body mass of about 100 g (Schlund, 2005). In Central Europe, this nocturnal rodent occurs preferentially in deciduous mixed forests dominated by the European beech (Schlund 2005). They produce one litter per year and after a gestation period of about 30 days, litters averaging 5-6 young are born in August (Fietz et al., 2009). For a small mammal, edible dormice are comparatively long-lived and may reach ages of up to 11 years with an average longevity of 3-4 years in nature (Ruf et al., 2006).

This study was conducted between 2012 and 2014 in south western Germany at five different sites in mixed deciduous forests, dominated by the European beech (Tab. 1). In each study site, nest boxes, that are used by dormice to rest during the day and to rear their offspring, were installed three meters above ground at the intersections of grid lines marking 30×30 m squares.

Table 1. Locations including coordinates, sizes of forests and study sites, number of individuals (except juveniles) captured during the whole study period (2012-2014).

Study site	Locations near the city	Coordinates	Forest size [ha]	Size of study site [ha]	Number of nest boxes	Total number of individuals captured in 2012 - 2014 (individuals/ha)
HE	Tübingen	48°33'03.38''N 8°59'59.72''E	15,000	20.4	228	14.1
KW	Ulm	48°22'56.50''N 9°55'54.45''E	135	7	70	18.7
BG	Ulm	48°25'22.36''N 9°57'43.17''E	70	7	70	64.9
BS	Ulm	48°29'21.18''N 9°58'219.02''E	33	7	70	41.1
JH	Ulm	48°22'11.13''N 9°49'3.44''E	11	7	70	26.6

2.2. Capture-mark-recapture & body measurements

We monitored nest boxes at all study sites from the end of May through September during daytime at bi-weekly intervals from 2012 through 2014. Upon first capture, dormice were individually marked using subcutaneously-implanted passive integrated transponders (Trovan, EURO I.D. Usling, Weilerswist, Germany). We recorded sex, assessed body mass for each capture to the nearest gram using a 300 g spring balance (Pesola, Baar, Switzerland; division: 2 g, accuracy: 99.7%) and measured tibia length and males' testes length to the nearest 0.1 mm using a sliding caliper. At the end of the procedure, animals were returned to their nest boxes. In males, reproductive activity was determined by checking for the presence of tangible testis. Females were classified as reproductively active for the respective study year, if they showed clear signs of gestation or lactation.

2.3. Urine sampling

Urine of dormice was sampled throughout the study, whenever an animal was urinating during the handling procedure. Samples were collected directly into 1.5 ml tubes and stored for maximally 8 hours in a cool bag, while being in the field and subsequently at -20 °C in the laboratory until further analyses.

2.4. Cort analyses

We determined cortisol concentrations in the urine, as urinary CORT levels correlate well with plasma free cortisol (Nakamura and Yakata, 1983) and corticosterone concentrations were shown to be negligible in this species (Havenstein & Fietz, unpublished data). CORT concentrations were determined radioimmunologically after extraction with ethyl acetate (Applichem, Darmstadt, Germany) as described by Wesoly et al. (Wesoly et al., 2015) for porcine samples, utilizing an antiserum raised in rabbits inoculated with cortisol-3-carboxymethyloxime conjugated to bovine serum albumin. The antiserum was used at a final dilution of 1:72000 in the assay. Cortisol-1,2,6,7-^{3}H with a specific activity of 72.4 Ci/mmol was purchased from Perkin Elmer (Waltham, MA, USA). The tracer was used in a dilution of 24000 dpm/100 µL in phosphate buffer (PB, 0.02 M KH_2PO_4, 0.059 M Na_2HPO_4, 5.4 mM NaN_3, Merck). The dose-response curve standards of cortisol (hydrocortisone, Sigma Aldrich Chemicals, Munich, Germany) ranged from 10 to 1000 pg/ 100 µL were prepared. Spiked samples for the determination of precision of the assay were prepared by adding known amounts of cortisol to urine with low endogenous cortisol concentration.

To correct for procedural losses, the recovery rate was determined with 3H-cortisol and averaged 89.3%. Precision was determined with spiked samples containing varying doses of unlabeled cortisol, revealing a mean recovery rate of 88.4%. Intraassay variation ranged between 6.0 % and 12.1 % in the three years, interassay variation ranged between 9.1 % and 27.8 %, depending on the concentrations of the samples. The lowest level of sensitivity ranged for an aliquot of 5 µl urine/assay between 5.5 and 7.9 ng/mL. The antibody HCO-21-HS SA1 cross-reacts with corticosterone (12.1 %), 21-desoxycorticosteron (6.8 %), aldosterone (1.2 %), progesterone (1.4 %), but not with estradiol or estrone. Cross-reactivity with pregnenolone was negligible (<0.01 %). Parallelism with the standard curve was demonstrated by serial dilution as well as serial volume multiplication of a urine sample.

For extraction, urine samples were diluted 1:40 and 1:20, depending on the concentration. 100 µL of diluted and spiked samples, respectively, were extracted with 2 mL of ethyl acetate (Applichem, Darmstadt, Germany) by mixing gently for 30 min. and subsequent freezing at -20°C for 1 h. The supernatants were collected and evaporated. The dried residues were resuspended in 100 µL PB.

For radioimmunoassay, 500 µL diluted antiserum and 100 µL of tracer were added to all biological and spiked samples, reagent blank and the standards. After incubation for 30 min. at 37 °C, the reaction was stopped on ice for 1 h. The bound-free separation was carried out by adding 500 µL of ice cold dextran-coated charcoal suspension, prepared by mixing 5 g of doubly washed charcoal (Serva Electrophoresis, Heidelberg, Germany) with 0.5 g of dextran 60 (Serva) and 1000 mL water, mixing for 1 min. and centrifugation for 20 min. at 3000 rpm, 4 °C. The supernatant containing the

antibody-bound cortisol was decanted into 5 mL of scintillation fluid (Lumasafe Plus, Perkin Elmer) and radioactivity was counted with a β-counter after a minimum of 30 min. (Beckman LS 1801).

2.5. Creatinine determination

To control for variations in fluid intake, CORT concentrations were corrected for creatinine content, applying a modified colorimetric end-point assay (Jaffe 1886, Folin 1904). After dilution of urine samples 1:10, 150 µL chromogenic substrate (8.73 mmol/l picric acid; 187.8 mmol/l NaOH; 7.5 mmol/l phosphate; Creatinin Jaffee Kinetic Fluid 1+1, mti-diagnostics GmbH, Idstein, Germany) was added, mixed, and after 10 min. incubation the extinction was determined with an automated microplate reader at 520 nm wavelength. A urine sample containing varying doses of appended creatinine (Merck, Darmstadt, Germany) served as quality controls. In this publication, the abbreviation CORT refers to cortisol corrected for creatinine (ng/mg).

2.6. Statistical Analyses

In accordance with the biology and physiology of the edible dormouse, we defined in males four and in females five distinct time periods during their active season: the post-hibernation period (PostH) starts directly when the first animals are observed after termination of hibernation at the end of May and lasts until the mating period (M, 25th June - 10th July). These two periods are identical for both sexes. In males, M is followed by the post-reproductive period (PR), and lasts until the pre-hibernation period (PreH, starting: 12th-20th September), which comprises the last 9 days before males enter hibernation. In females, the mating period is followed by the gestation-lactation period (GL, 30th July -29th August), that starts around mid-gestation and encompasses the time of lactation. When juveniles are weaned, a short post-reproductive period (PR) starts for females, followed up by PreH (Julian Day: 12th-20th September). Note that in edible dormice reproduction is synchronized with the masting pattern of the beech. Therefore, the factor ‘‘food/ repro” can adopt two levels: “high” means that food availability is high and virtually all dormice reproduce within this year (2013 and 2014), whereas “low” means, that beeches and dormice faile to reproduce (2012). Data obtained during 2012 in BS were attributed to a high food/repro year as masting oak trees provided high quality food and dormice reproduced. We analyzed the seasonal variation in CORT levels in high as well as low food/repro years, separately for males and females and investigated sex effects by focussing on the respective periods of high reproductive investment, M and GL. To investigate the effect of food on HPA axis activity, we compared CORT values of males achieved when seeds are ripe (August and September) in high food years, with values obtained during the same time period in the year of mast failure. In females it is impossible to disentangle the effects of food availability and reproductive investment. To examine the effect of reproduction on CORT levels, we compared CORT

concentrations of both sexes during the reproductive periods between high repro years and the respective time period in a low repro year. To elucidate effects of the year in two consecutive reproductive years and of the study site, we separately analyzed PostH (as PostH represents a critical period in the life of dormice (Havenstein et al., 2016; Lebl et al., 2011) and the periods of high reproductive investments of high food/repro years.

We used R 3.2.3 (R Core Team, 2015) and the packages lme4 and lmerTest (Bates et al. 2015; Kuznetsova et al., 2015) to perform linear mixed effects analyses of the relationships between CORT levels and biologically relevant fixed effect variables ‘‘period‘‘ (males: PostH, M, PR, PreH ; females: PostH, M, GL, PR, PreH; see above), ‘‘study site‘‘ (five levels according to the different study sites), ‘‘body mass‘‘ and ‘‘food/repro‘‘ (two levels: low and high) or ‘‘study year‘‘ (two levels 2013/2014). We further included ‘‘individual” as random effect. Study site, body mass, or study year were successively excluded from the final model if they were non-significant and their exclusion improved the model according to the AIC. Data were transformed if necessary to achieve normality and homoscedasticity. The function summary were used to obtain results of the models, the Satterthwaite approximation for degrees of freedom was used to calculate p-values. Test results were considered significant, if p was <0.05. Details of the particular models used are given within the respective results section. In figures, asterisks are used to display significance levels according to MMs with the following indications: .p < 0.1; *p < 0.05; **p < 0.01; ***p < 0.001.

3. Results

3.1. Urine samples collected

We collected 391 urine samples of 281 individuals (153 females and 128 males). 94 samples (44 females, 40 males) were collected within a low food/repro year (2012, except for BS), 297 samples (99 females, 84 males) were obtained during high food/repro years.

3.2. Seasonal variations and effects of reproductive activity on stress hormone levels

Males

In high food/repro years, males emerged from hibernation with relatively low CORT concentrations that increased significantly during the following mating period (M), fell below PostH values during the PR period and rose again to levels comparable to mating values during PreH (Fig. 1, Table 2). In the second year of the two consecutive reproductive years (2013 & 2014), CORT concentrations were in general significantly higher than during the first reproductive year (Table 2).

During the low food/repro year, CORT concentrations of males were highest during PostH. During the subsequent periods (M and PR), CORT ranged on lower levels, but it increased again towards the end of the active season (PreH; Table 2, Fig. 1).

CORT levels were significantly higher during the mating periods of reproductive years, than during the same time period of a low food/repro year (Model: M: logCORT= $year_{13}$ + body mass + ID random; n=54; DF=54; $Estimate_{14}$=-1.038, SD=0.205, p<0.001; $Estimate_{body\ mass}$ =-0.014, SD=0.005, p=0.004, Fig. 1).

When comparing CORT levels of two consecutive reproductive years , CORT concentrations of males in the second reproductive year were lower during PostH, but higher during M (Model: PostH: logCORT= $year_{13}$ + study site + ID random; n=26; DF=20; $Estimate_{14}$=-0.816, SD=0.327, p=0.021; M: logCORT= $year_{14}$ + ID random; n=31; DF=28.9; $Estimate_{14}$=0.869, SD=0.412, p=0.043).

Females

In females CORT concentrations were lowest at the beginning of their active season in high food/ repro years (Fig. 1), elevated during M and increased further during the period of lactation and gestation (Fig. 1). During the following post-reproductive period, CORT concentrations dropped to levels similar to mating CORT levels and increased again before females entered hibernation. Also in females, CORT concentrations were generally higher in the second of the two consecutive reproductive years (Table 2).

CORT concentrations during summer of the low food/repro year were significantly lower compared to PostH in high food/repro years, the period with the lowest CORT levels of the reproductive years (Model: logCORT= $Period_{PostH\ high}$ + study $site_{BG}$ + ID random; n=26; DF=26; $Estimate_{summer\ low}$=-0.66, SD=0.25, p=0.015; $Estimate_{HE}$ = 0.97, SD=0.29, p=0.002). In the low food/ repro year female CORT concentrations ranged on comparable levels during M and GL, but augmented during the last two periods (PR and PreH; Table 2, Fig. 1).

CORT levels measured in females during M and the following GL period were distinctly higher than during the corresponding periods in a year of reproductive failure (Models: M: logCORT= $food/repro_{high}$ + body mass + ID random; n=121; DF=97.83; $Estimate_{high}$=0.721, SD=0.213, p=0.001; $Estimate_{body\ mass}$= -0.014, SD=0.005, p=0.008; GL: logCORT= $food/repro_{high}$ + ID random; n=106; DF=105.81; $Estimate_{high}$=1.45, SD=0.235, p<0.001, Fig. 1).

Table 2. Estimates of fixed effects of the general linear mixed model for CORT concentrations of male (left) and female (right) edible dormice collected in all study sites during the entire active period of a low food/repro year (2012, upper tables) and the high food/repro years (2013 and 2014, lower tables). Model low food/repro year: log CORT = period + study site+ ID random, (males n=46, females n=48). Model high food/repro years: log CORT = period + year+ study site + ID random, (males n=123, females n=158). References for comparison were PostH/period, BG/study site, 2013/year. In the low food/repro year no urine samples of females were obtained during PostH, therefore the reference period is M for this analysis.

2012, low food/repro

		Males						Females				
		Estimate	SE	DF	t	p			Estimate	SE	DF	t	p
Intercept		3.138	0.46	38	6.86	<0.001	Intercept		3.004	0.18	46	17.10	<0.001
Period	M	-0.601	0.39	39	-1.53	0.135	Period	GL	-0.248	0.19	42	-1.31	0.199
	PR	-0.208	0.39	39	-0.53	0.600		PR	0.383	0.28	41	1.39	0.171
	PreH	1.373	0.47	35	2.93	0.006		PreH	1.098	0.41	14	2.70	0.017
Study Site	JH	0.361	0.32	39	1.13	0.267	Study Site	JH	0.100	0.24	42	0.42	0.677
	SF	0.963	0.34	38	2.82	0.008		SF	0.190	0.27	41	0.70	0.491
	HE	0.759	0.34	38	2.22	0.033		HE	1.281	0.24	42	5.32	0.000

2013/14, high food/repro

		Estimate	SE	DF	t	p			Estimate	SE	DF	t	p
Intercept		3.131	0.2	114	15.75	<0.001	Intercept		3.199	0.29	155	11.09	<0.001
Period	M	0.570	0.17	107	3.39	0.001	Period	M	0.048	0.29	155	0.16	0.871
	PR	-0.541	0.16	123	-3.45	0.001		GL	0.759	0.28	159	2.73	0.007
	PreH	0.212	0.29	123	0.72	0.474		PR	0.633	0.32	160	1.97	0.051
								PreH	0.912	0.32	160	2.85	0.005
Year	2014	0.466	0.13	120	3.73	0.000	Year	2014	0.309	0.14	160	2.23	0.027
Study Site	BS	0.626	0.23	95	2.75	0.007	Study Site	BS	0.383	0.25	120	1.53	0.129
	JH	0.221	0.22	100	0.99	0.326		JH	0.639	0.24	135	2.67	0.009
	SF	0.732	0.25	100	2.92	0.004		SF	0.459	0.24	119	1.95	0.054
	HE	0.287	0.22	102	1.28	0.202		HE	0.883	0.22	123	3.99	0.000

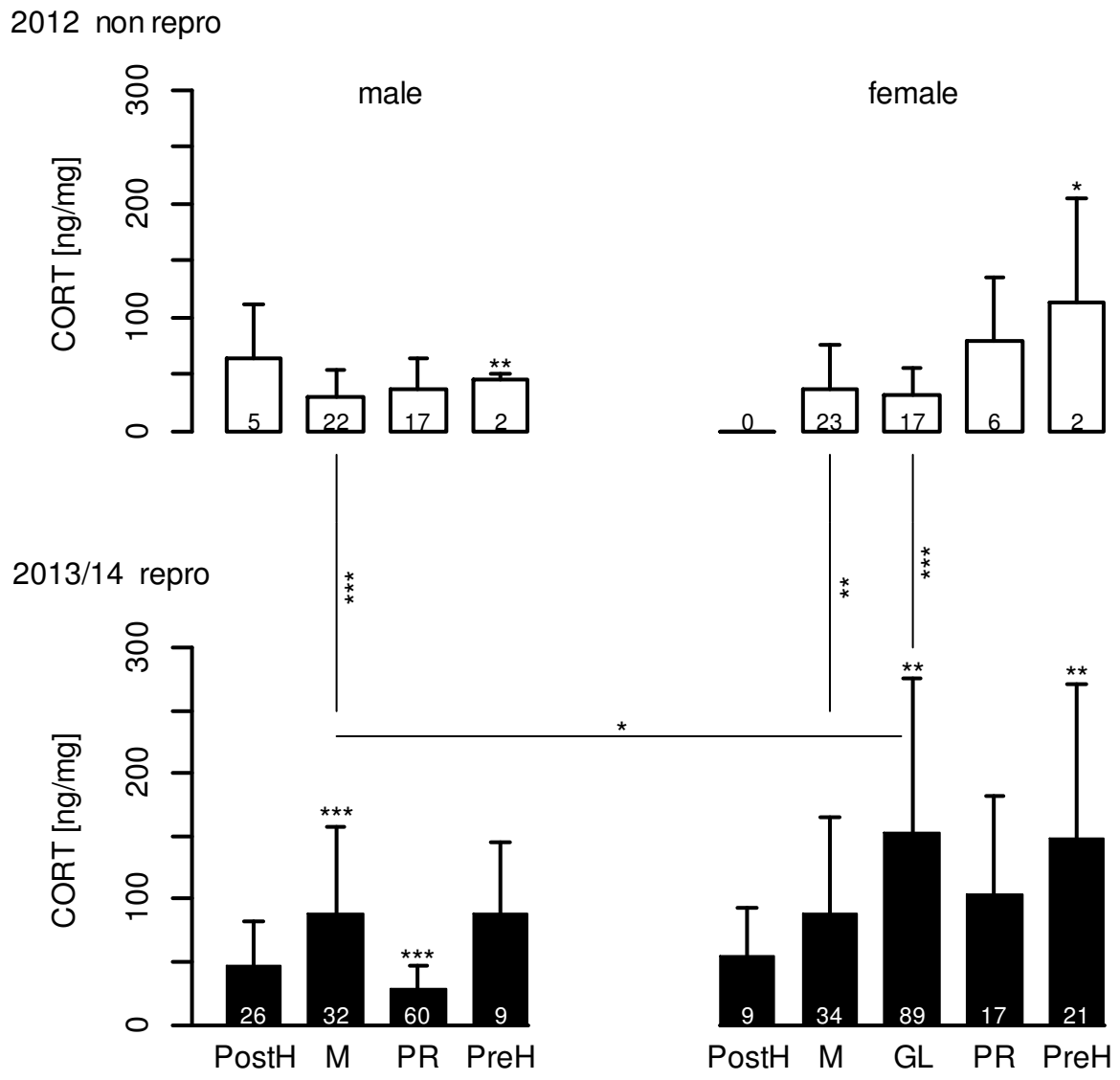

Fig. 1 Seasonal changes of cortisol concentrations in the urine in male (left) and female (right) edible dormice during their active period in the low food/repro year 2012 (top) and the high food/repro years 2013 and 2014 (below) (mean and SD). Reference for variations among periods within the sex and the food/repro year was PostH, except for the females in the low food/repro year, where M serves as the reference due to the late emergence date of the females during this year.

3.3. Comparison of males and females

Males and females did not differ in their CORT levels during the mating period (Model: logCORT=sex_m + body mass + ID random; n=66; DF=66; $Estimate_{male}$=0.063, SD=0.196, p=0.75, Fig. 1) but body mass was negatively correlated with CORT concentrations ($Estimate_{body\ mass}$=-0.014, SD=0.0049, p=0.0056). When comparing CORT levels of males during mating to those of females during gestation and lactation, we found higher CORT levels in females (Model: logCORT=sex_{female} + ID random; n=121; DF=97.83; $Estimate_{male}$=-0.464, SD=0.198, p=0.021, Fig. 1).

CORT levels did not differ among sexes under low food availability (Model: logCORT=sex_m + body mass + ID random; n=36; DF=36; $Estimate_{male}$=-0.066, SD=0.273, p=0.81) but individuals with a higher body mass had lower CORT concentrations ($Estimate_{body\ mass}$=-0.014, SD=0.006, p=0.039).

3.4. Effects of food availability

CORT values of males measured during August and September in a high food year did not differ from those measured during the same time period of a low food year (Model: logCORT= food/$repro_{high}$ + ID random, n=66, DF=64.34, p=0.422).

3.5. Study site effects

In all study years, males from BG had lowest CORT values. During reproductive years, the differences were significant for BS and SF (Table 2, Fig. 1), in the non-reproductive year, the difference was significant for SF (Table 2, Fig. 1).

In females, we found that in all years CORT levels were significantly lower in BG (highest population density) compared to HE, as well as to JH during reproductive years (Table 2, Fig. 1). When analyzing the study site effect explicitly during GL of reproductive years, we found that CORT values in BG were lowest compared to all other sites. The differences were most prominent among BG and HE, followed by JH, BS, and SF (Table 3).

Table 3. Estimates of fixed effects of the general linear mixed model for CORT concentrations of female edible dormice collected in all study sites during GL of the high food/repro years (2013 and 2014). Model: log CORT = study site + ID random, (females n=89). Reference for comparisons was BG/study site.

		Estimate	SE	DF	t	p
Intercept		3.849	0.222	76	17.36	<0.001
Study Site	BS	0.844	0.301	68	2.80	0.007
	JH	1.034	0.331	64	3.12	0.003
	SF	0.756	0.307	69	2.46	0.016
	HE	1.103	0.296	67	3.72	0.000

4. Discussion

4.1. Seasonal variations in CORT levels in males & females

Edible dormice exhibit strong seasonal variations in CORT levels with peaks in association with reproductive activity and pre-hibernation. Elevated CORT levels detected during the mating season in males and during gestation and lactation in females indicate that reproduction represents a

demanding, potentially stressful event for both sexes. Stress-induced high GC concentrations might prevent reproduction by suppressing the activity of the hypothalamic-pituitary-gonadal (HPG) axis (Kirby et al., 2009; Nakamura et al., 2008). In our study, elevated CORT levels in males coincided with the mating season, when levels of testosterone are high (Jallageas and Assenmacher, 1983). Therefore, we assume that high stress hormone-mediated repression of reproductive function in males can be excluded. Edible dormice exhibit a promiscuous mating system and juveniles belonging to the same litter may be sired by multiple males (Fietz, unpubl. data). Multiple paternities, a significant body mass loss in reproductively active males (Fietz et al., 2009) and scars found in older males (Ruf et al., 2006) suggest a strong intrasexual competition of males for receptive females, supporting the notion that the mating season is a stressful period for dormice. In both sexes, higher body mass is associated with lower CORT levels during the mating season. This relationship has often been found in wild mammals (Haase et al., 2016), suggesting that individual traits like body condition play a pivotal role in HPA axis functionality and the perception of stress (see also McEwen and Wingfield, 2003). It is important to note that a HPA-HPG axis crosstalk also operates reversed, namely that high testosterone levels inhibit HPA-axis activity, which serves to keep CORT concentrations within physiological baseline ranges supporting reproduction. This interrelation is well examined in laboratory rodents and has also been shown in the wild (yellow-pine chipmunks, *Tamias amoenus,* Belding's ground squirrels, *Spermophilus beldingi;* Handa and Weiser, 2014; Nunes et al., 2006; Place, 2000) and may explain moderately elevated CORT levels in male dormice during the mating season compared to the extreme increase in females during gestation and lactation. Thus, during mating an interaction of stress-induced CORT level increases and a concurrent down regulation through sex hormones presumably determine the moderate increases in CORT concentrations in males which seems to represent a coping mechanism to facilitate reproduction while complying with the demands of the mating activities (see also enabling hypothesis of CORT; Fletcher et al., 2015; Landys et al., 2006).

Gestation and lactation represent the most demanding periods in the life time of a female (Speakman, 2008; Wade and Schneider, 1992). We found slightly elevated CORT levels during mating that increased extensively during the subsequent gestation and lactation period. The gestational hormones estrogen and progesterone are known to cause considerable baseline CORT increases and are assumed to be necessary for mediating the shift to the primarily catabolic metabolism towards the end of pregnancy to meet fetal demands (Altemus et al., 1995; Atkinson and Waddell, 1995; Handa and Weiser, 2014; Reeder et al., 2004). Contrary to the inhibiting impacts of androgens on HPA axis activity, findings from ovariectomized rats and estradiol-progesterone treated rats reveal that estrogens enhance the HPA axis responsiveness to stress (Handa and Weiser, 2014), further

explaining the observed discrepancy in male and female CORT increases during their respective reproductive periods of our study. During lactation, however, a decrease in CORT levels even below pre-gestational values has been shown in various species, which may serve to save energy for milk synthesis and to down-regulate anxiety of the mother, as a high anxiety level could negatively affect the raising of the offspring (Altemus et al., 1995; Atkinson and Waddell, 1995; Reeder et al., 2004). In general, reproducing individuals seem to be less sensitive towards stress, resulting in diminished HPA responses to stressors and consequently mitigated physiological or behavioral effects (McEwen and Wingfield, 2010; Wingfield and Sapolsky, 2003). In our study, CORT concentrations not only of gestating but also of lactating females ranged on distinctly higher levels compared to pre-reproductive periods and compared to CORT concentrations of males during the mating season. These high levels stand in contrast to the usual stress hormone pattern and stress sensitivity of other lactating mammals and strongly suggests that dormouse females bear an extremely high reproductive burden during lactation caused by high energy demands and reduced time for foraging. In female tree swallows (*Tachycineta bicolor*) intensified reproductive effort provoked by experimentally increased clutch sizes were positively correlated with CORT levels (Bonier et al., 2011). As a higher number of offspring fledged, the authors argued that the increased CORT concentration enabled the females to comply with the artificially elevated parental demand. In dormice, we rarely observed litters where the mother had died during lactation. Accordingly, we assume that high CORT levels in females during lactation, in the first place, support individuals to cope better with the extreme challenge of lactation and facilitate offspring raising (Fletcher et al., 2015). However, additional physiological examinations revealed negative consequences of reproduction for the condition of edible dormice (see below). Furthermore, in contrast to the findings in males, elevated post-reproductive CORT levels indicate that females obviously still incur an elevated allostatic load and do not recover immediately after reproduction even though food is highly abundant after weaning.

After termination of mating activities CORT levels in males dropped sharply to lowest average CORT levels, indicating that this period, when high quality food is vastly available, is the least demanding period for male dormice. Accordingly, CORT concentrations measured during this period constitute basal levels, i.e. levels essential to maintain basic body functions in terms of homeostasis, like the provisioning of glucose to cells, in absence of a demanding situation (e.g. Landys et al., 2006). In females, CORT concentrations also decreased after reproduction, but the lowest stress hormone concentrations that may constitute basal levels occurred during summer of a non-reproductive year. Thus in reproductive years, all periods of the active season obviously entail elevated demands for females.

In all study years, we detected a CORT level increase shortly before the onset of hibernation well after reproductive activity was terminated. Besides the arctic and the golden-mantled ground squirrels (*Spermophilus parryii, Callospermophilus lateralis)* where no pre-hibernation increases were detected (Michael Romero, 2002), elevations in GC levels prior to hibernation were observed across different species, e.g. the yellow-bellied marmot (*Marmota flaviventris*, free-ranging; Armitage, 1991), the European ground squirrel (*Spermophilus citellus*, captive; Shivatcheva et al., 1988), the European hedgehog (*Erinaceus europaeus*, captive; Saboureau et al., 1980), and the little brown bat (*Myotis lucifugus*, free-ranging*;* Reeder et al., 2004). In addition to their catabolic effects, GCs are also known to induce anabolic actions, including an increase of food intake, hyperphagia, and fattening (Dallman et al., 1995). These relationships suggest that GC elevations prior to hibernation may promote pre-hibernation fattening or alternatively, support the entrance into hibernation, e.g. by mediating the switch from glucose to fatty acid metabolism (Carey et al., 2003).

After emergence from hibernation, hibernators have to invest into the restoration of regressed organs, such as the digestive system and the gonads (Carey et al., 2003; Kruman, 1992). In the edible dormouse, this restoration occurs when high quality food is still missing (Carey et al., 2003; Schlund et al., 2002). A decrease in body mass (Fietz et al., 2004) at the beginning of the active season together with elevated CORT levels, especially in reproductive years, indicate that dormice are in a negative energy balance and suggest that elevated CORT levels serves to mobilize energy for these restoration processes.

4.2 Reference to further physiological parameters

In wildlife studies it is hard to disentangle adaptive physiological increases in CORT concentration from a stress response upon noxious stimuli and evaluate the respective impact on the performance of the individuals. Therefore, it is recommendable to examine additional health- or fitness-related variables (Bonier et al., 2009; Dantzer et al., 2014; Davis et al., 2008; Wikelski and Cooke, 2006). Studies on wild birds at tourist-exposed sites revealed an association between high GC levels and increased mortality in hoatzin chicks (*Opisthocomus hoazin,* Müllner et al., 2004) and a reduced number of fledglings in yellow-eyed penguins (*Megadyptes antipodes,* Ellenberg et al., 2007). In a previous study, we revealed impairments in the oxygen carrying capacity during reproduction in the same populations during the same time period that are like the CORT level increases considerably more pronounced in females (Havenstein et al., 2017). Although high stress hormones are not the main driver for the impairments in the oxygen delivery system the elevated CORT levels presumably

exacerbate the reduction in erythrocyte numbers by provoking an increase in oxidative damage to the RBCs. The erythropoiesis-boosting capacity of high CORT levels obviously take effect in males but is impeded by gestational hormones in females (Fibach and Rachmilewitz, 2008). Hence, high CORT levels presumably contribute to a certain extend to a better performance and recovery, especially in males and in females after gestation (Havenstein et al., 2017). Concurrently, a physiological stress response was observed on the immune system level (Davis et al., 2008; O'connor et al., 2000; Shi et al., 2003; Wang et al., 2002), namely an increase in the P/L ratio (phagocyte: lymphocyte counts; Havenstein et al., 2016) which is likely to hallmark the beginning of stress-induced health deterioration. We showed that these physiological alterations persisted for an extended time period or even exacerbated during a consecutive reproductive season, which indicates prolonged effects of stress (Havenstein et al., 2016; Havenstein et al., 2017). Furthermore, high reproductive investment results in increased mortality rates in edible dormice (Ruf et al., 2006) that can now be linked to these physiological impairments and high stress hormone levels. These findings indicate that CORT concentrations during reproduction surpassed physiological thresholds of adaption-mediating levels and represent a situation of allostatic overload (e.g. McEwen and Wingfield, 2010) mediating a trade-off of reproduction for survival (Harshman and Zera, 2007; Stearns, 1989). Furthermore, in the annual cycle of edible dormice mortality is highest after emergence from hibernation (Lebl et al., 2011) when regressed organs and functions require restoration (Carey et al., 2003), food availability is limited and the immunological first line of defence is deteriorated (Havenstein et al., 2016). Accordingly, the post-hibernation period obviously represents a vulnerable period for dormice which is not reflected by drastically increased CORT levels. Thus, CORT concentrations alone cannot always serve as an indicator for fitness-depleting conditions.

4.3 Negative vs. positive carry-over effects

The post-hibernation vulnerability may not only result from effects of hibernation but also from demanding pre-hibernation situations, like prolonged food limitation or high reproductive investment. Time-lagged effects of reproduction on fitness were also suggested in other wild mammals. In cooperatively breeding mongooses (*Mungos mungo*), reduced parental investment related to preceding high GC levels, especially if a short time period passed between two breeding attempts (Sanderson et al., 2014). Experimentally increased brood sizes in kestrels (*Falco tinnunculus*) resulted in an increased mortality half a year later (Daan et al., 1996) but the underlying mechanism remained unknown. In edible dormice, despite the decline in stress hormone concentrations after termination of the demanding reproduction, CORT level increases and impairments in oxygen delivery capacity during reproductive activities amplify in a consecutive reproductive year, indicating an accumulation of allostatic load (McEwen and Wingfield, 2010, 2003).

This finding suggests prolonged effects of the stressful reproductive activities on the HPA axis and the physiology of edible dormice. Hence, the stress of reproduction, the need to restore the oxygen delivery capacity thereafter and the prolonged immunological impairment may result in a negative carry-over effect contributing to an impaired performance during PostH when individuals are again in a vulnerable condition for a poor energy status.

However, the analysis of two consecutive reproductive years revealed lower CORT levels at the beginning of the second reproductive year. In edible dormice and in alpine marmots (*Marmota marmota*) a higher accumulation of body fat before body mass at immergence into hibernation results in longer arousal phases and shallower torpor bouts, especially towards the end of hibernation (Arnold and Dittami, 1997; Bieber et al., 2014). Late arousals are used for the physical preparation for the active season (see e.g. Carey et al., 2003), the extend of preparation we previously suggested in dormice to be adjusted to expected upcoming food availability and reproductive effort (Havenstein et al., 2016). However, larger fat reserves may enable them to utilize late arousals more intensely for restoration processes (Carey et al., 2003; Kruman, 1992). An advanced restoration upon emergence implies a reduced need to search for food when food availability is limited and reduced restoration processes, explaining lower CORT concentrations, indicating a positive carry-over effect of high food availability. Thus, the allostatic load dormice experience after termination of hibernation is a result of expected upcoming effort (reproduction or no reproduction) and pre-hibernation conditions (food availability and reproduction).

4.4 Food availability

The availability of food or body energy reserves is an important determinant for the ability to cope with allostatic load in demanding situations (Landys et al., 2006). Increased GC levels in situations of low food availability may hence represent high psychological-nutritional stress or result from the physiological mechanism to mobilize fuel during fasting periods (Sapolsky et al., 2000). High GC levels under restricted food availability have been found for example in killer whales (*Orcinus orca,* Ayres et al., 2012) and common guillemots (*Uria aalge*, Barrett et al., 2015). However, the predictability of stressors and demanding situations seems to be pivotal for the resulting allostatic load (McEwen and Wingfield, 2010). Accordingly, fasting associated with migration in birds or with egg-incubation in penguins do not induce excessively elevated CORT levels, presumably because individuals anticipate these fasting periods that belong to a particular life history stage (Landys-Ciannelli et al., 2002; Vleck and Vleck, 2002). Food availability seems to be predictable for edible dormice resulting in reduced energy expenditure in years of low food availability (Langer et al., unpubl. data). In accordance with this assumption, we could not detect a difference in CORT levels of individuals under low and high food availability, respectively. Thus, these extended fasting periods do not impose stress on our

study species, in contrast, the "energy saving mode" efficiently reduces energy expenditure without the need to extensively mobilize fuel from internal energy stores. This is in line with our previous results, showing that the P/L ratio is not considerably elevated during fasting (Havenstein et al., 2016). Obviously, the edible dormouse is perfectly adapted to cope with extended periods of fasting and as long as individuals are in a good body condition, they will overcome these challenges by adjusting their energy expenditure.

4.5 Study Site- Effects

Except for the PostH period in males, CORT levels were lowest in the study site with the highest population density (BG), irrespective of reproductive activity and food availability. These results correspond well to our previous findings on impairments in the P/L ratio and the oxygen delivery system (Havenstein et al., 2016; Havenstein et al., 2017). This consistent pattern of different physiological parameters clearly indicates a lower level of stress and physiological demand in animals from this site. Thus, high population density does not entail elevated adverse effects through competitive encounters for food and shelter. Conversely, high population density is rather the result of favorable environmental conditions. Consequently, other factors than population density affect individual performance. In small mammals predation is the main cause of mortality (Bryant and Page, 2005; Ims and Andreassen, 2000) and may have a strong impact on population densities as well as on the perceived stress level of individuals. This could be convincingly demonstrated in snowshoe hares, where reproduction was effectively suppressed during and even some years after high predation pressure (Sheriff et al., 2009). Augmentations in reproductive effort and spatial variation in food availability can also alter HPA axis functioning distinctively (Boonstra et al., 2014). Furthermore, logging and destruction of habitat represent unpredictable, potentially life-threatening disturbances especially for small arboreal animals, as they may directly kill individuals (Escobar et al., 2015) or may alter the habitat strongly concerning food sources, predation risk (Bryant and Page, 2005) and shelter. This applies particularly to species that use tree holes for resting and raising their offspring such as edible dormice (Meijaard et al., 2005). Anthropogenic disturbance through logging was shown to increase GC levels, for example, in brown spider monkeys (*Ateles hybridus,* Rimbach et al., 2013), spottet owls (*Strix occidentalis,* Wasser et al., 1997) and eastern chipmunks (*Tamias striatus,* Mastromonaco et al., 2014). Among our study sites, BG is the only site that is not regularly logged for economic purposes. Absence of logging may be the ultimate reason for the extreme well performance in terms of stress hormone levels, body masses (Fietz and Weis-Dootz, 2012), P/L ratios (Havenstein et al., 2016), the oxygen delivery system (Havenstein et al., 2017) and high population density (Fietz and Weis-Dootz, 2012) that we encounter in this study site. But there seem to exist

further site-specific features that shape individual performance and demographic development which deserve identification.

Interestingly, during PostH males from BG showed higher CORT concentrations compared to all other study sites. In former studies, we further encountered highest values in haemoglobin concentration and haematocrit and signs of an elevated erythropoiesis in males of BG during this period of time (Havenstein et al., 2017). In line with our assumption of a preparative investment before and directly after emergence from hibernation we presumed that males in BG invested stronger into RBC generation directly after hibernation. The stress hormone pattern supports this notion as the slightly higher CORT concentrations in these males may indicate an elevated provision of energy. Obviously, males from BG can derive advantage from their larger body masses (Fietz and Weis-Dootz, 2012) and use surplus energy to quickly restore organs and physiological functions early in the active season to rely on a good body constitution during the reproductive period. These slightly different physiological patterns observed among the study sites demonstrate a variation in coping strategies in dependency of environmental conditions.

5. Conclusions

The level of cortisol in wild edible dormice is influenced by various effectors, including reproduction, hibernation, study site as well as sex, body condition, and study year. Our findings indicate that increases in CORT concentrations generally reflect the adjustment of the hormonal set point to current demands and hence mediate enabling functions. Accordingly, stress hormone levels indicate situations of elevated demand and metabolic requirements, emphasize the ability of dormice to cope with food restriction and give notice of favourable environmental conditions at the study site with the highest population density which is in line with further findings on immune and red blood cell parameters. Strongly increased CORT levels during reproduction are associated with impairments in the immune and the oxidative system and can be linked to prolonged and exacerbated physiological impairments as well as increased mortality after a reproductive season. These findings indicate that the stress level and the demands of reproduction exceed physiological ranges and render predictive power to this measure. However, during the post-hibernation period, when mortality is highest in the annual cycle of dormice, CORT concentrations range on only moderately elevated levels. Hence, pure CORT concentrations do not always relate to fitness-depleting conditions. Therefore, to evaluate the condition of individuals and investigate the mechanisms underlying fitness deprivations, the prolonged investigation of stress hormone levels combined with further physiological indices are needed. Thus, an in depth analysis of CORT levels, life conditions, and additional physiological

measures help to disentangle the impact of different stressors and to link them to associated physiological as well as ultimately fitness trade-offs.

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Chapter V: General Discussion

The life cycle of wild animals is subject to diverse, and often coincidentally occurring challenges caused by environmental influences and demanding life history stages. As organisms perform multiple costly body functions while resources are limited, certain situations may entail allocation decisions resulting in physiological trade-offs among different processes. Such trade-offs potentially impair performance and ultimately fitness of an individual. In the light of worldwide population declines and species extinction, a major goal in ecology is to elucidate how different challenging situations impact physiological systems and fitness parameters. In this respect, the monitoring of glucocorticoid (GC) concentrations in wild populations is a powerful tool, as these hormones represent important mediators between the environment and the physiological responses of an organism that largely contribute to the regulation of most allocation decisions concerning growth, energy storage and reproduction (Harshman and Zera, 2007; Lee, 2006; Stearns, 1989). However, elevations in GC levels may represent physiological fluctuations in response to increased demands, helping the individual to cope with the current situation, or they may constitute a stress response upon noxious stimuli with potentially negative effects on the performance of the individual, if perceived for prolonged times (Landys et al., 2006). Therefore, it is recommendable to investigate further body functions that are vital for the organism and are known to respond sensitively to stressors and changed conditions, like e.g. metabolism, additional endocrine regulators, immunity and according to our opinion, also the oxygen delivery system (Lochmiller and Deerenberg, 2000; Ricklefs and Wikelski, 2002; Wikelski and Cooke, 2006).

Effects of Reproduction

Results of this study reveal significant effects of reproduction, hibernation, food restriction and study sites on GC levels, the immune system and the oxygen delivery system. Reproduction turned out to be an extremely demanding and stressful life history event in both sexes of edible dormice that entails considerable physiological impacts, involving impairments in the oxygen delivery system as well as marked increases in the CORT concentration that obviously lead to a rise in the P/L ratio. In both sexes, CORT levels fall after high reproductive investment, which is in males directly after the mating season and in females towards the end of lactation when juveniles start to feed on their own. In males, CORT levels of the post-reproductive period appear to represent basal stress hormone levels, i.e. levels in absence of a stressor or demanding condition that are necessary to maintain basic body functions in terms of homeostasis, like the provisioning of glucose to cells (e.g. Landys et al., 2006). In females, the lowest stress hormone concentrations that may constitute basal CORT levels did not occur after termination of reproductive activity but during late summer of a non-reproductive

year when dormice face prolonged periods of limited food availability. Due to gestation and lactation the effort of reproduction lasts considerably longer in females than in males and seems to impact the females more sustainably. Accordingly, the declining CORT levels during the short post-reproductive period of females still remain above post-hibernation CORT levels and well above the apparently basal CORT levels during late summer of a non-reproductive year. During periods of reproductive activity, increases in CORT concentrations as well as reductions in RBC counts and haemoglobin concentrations were considerably more pronounced in females than in males. This can partially be explained by the mode of action of the sex steroids: androgens generally suppress HPA axis activity, whereas estrogen and progesterone elevate baseline CORT levels as well as the HPA axis response upon stress and are furthermore known to depress erythropoiesis, explaining the observed differences between males and females during their respective period of high reproductive investment. Besides the differential influences of the sex hormones, the impairments in the oxygen delivery system observed during reproduction in both sexes seem to be caused to a large extend by energetic and nutrient deficits. Erythrocyte indices and a high frequency of haemoglobinuria in females point to the manifestation of a folic acid anaemia (high MCV and MCH) which is a widespread symptom during reproductive activity caused by high sex hormone levels and increased nutritional requirements while high quality food is still missing (Chapter III, Fig. 1 + 3, section "Seasonal variations in RBC indices"; Blobel and Orkin, 1996; Dukes and Goldwasser, 1961). Under a lack of folate, DNA synthesis is impaired while cell growth and protein synthesis continues, resulting in the liberation of few, large RBCs from the bone marrow that are packed with haemoglobin (high MCH). Due to their abnormal shape these RBCs are prone to haemolysis, that potentially leads to haemoglobinuria. Further mechanisms presumably contribute to the haematological changes in both sexes. Before and during the mating season, the lack of high quality food is accompanied by a high activity level, especially in males, as formerly indicated by body mass decreases (Fietz et al., 2004). Strong exercise as well as high stress levels are known to exacerbate oxidative damage and to shorten the life span of RBCs (Costantini et al., 2011; Jarolim et al., 1990; Mairbäurl, 2013; Santos-Silva et al., 2001). The negative energy balance presumably requires the allocation of resources away from RBC generation into reproductive activities, restricting erythropoiesis (Borelli et al., 2007; Cox et al., 2010; Kalmbach et al., 2004). Furthermore, the extended hibernation period obviously leads to an accumulation of senescent RBCs even though erythropoiesis seems to occur during arousals (see Chapter III, Table 3, section "Timing of RBC death and renewal"; Carey et al., 2003; Kruman, 1992; Lyman et al., 1957). As a result of these coinciding impacts the rate of RBC destruction exceeds the production during the reproductive period. However, the impairment in the oxygen transport capacity in males is moderate, presumably because the high CORT levels effectively display their erythropoiesis-boosting effect, as shown by increases in RBC size (MCV) and size variation (RDW) and

decreases in the MCHC, that represent the typical indicators of a regenerative response of the bone marrow to fill up the RBC pool (Lodish et al., 2010; Tyler and Cowell, 1996). Thus, we suggest that high CORT levels in males eventually contribute to a fast recovery and prevent an extreme shortfall in RBC numbers whereas the hormonal status of females prevent the positive effects of high CORT levels and primarily lead to increased RBC destruction (Keohane et al., 2015; Sivilotti, 2004).

Impairments in the oxygen transport system recover to a large extend after mating in males and gestation in females, respectively. The fact that females recover from the anaemia during lactation when CORT concentrations remain on high levels while estrogen levels are low and high quality food is vastly available further supports the notion of a gestation-associated folate-deficiency anaemia. Under these changed conditions, the high stress hormone levels possibly also contribute to the recovery from anaemia in females. The sustained high stress hormone level in lactating females contradicts the pattern found in other species during lactation, where GC levels usually decrease below pre-gestational levels (Altemus et al., 1995; Atkinson and Waddell, 1995; Reeder et al., 2004). This deviation assumably indicates the enormous demands imposed on dormouse females during offspring raising (see also Chapter IV, Fig. 1, section "Seasonal variations in CORT levels in males and females"). In both sexes, during the periods of high reproductive investment the white blood cell differential shows distinct increases in the P/L ratio, an immunological stress response. It has been suggested that these alterations in the immune cell counts might represent an enhanced immunity through a redistribution of immune cells to sites of pathogen encounter or a reallocation of immunological power towards innate immune defences (Dhabhar and McEwen, 1997). However, high CORT levels responsible for these alterations also induce lymphocyte apoptosis and down-regulate neutrophil diapedisis, which hampers migration of neutrophils to sites of inflammation (O'connor et al., 2000; Wang et al., 2002). Furthermore, clinical investigations reveal a correlation between high neutrophil/lymphocyte ratios and exacerbation of disease development (Kocyigit et al., 2013; Liu et al., 2015) and the negative impacts of chronic stress on immunity and health are well examined (Glaser and Kiecolt-Glaser, 2005; Webster Marketon and Glaser, 2008). Accordingly, the observed increase in the P/L ratio presumably hallmarks the beginning of stress-induced health deterioration. In line with these observations P/L_{prop} ratios measured in reproductive males during the mating season were significantly elevated in comparison to those measured in sexually quiescent males during the same time period in the low food/repro year (Chapter II, Fig. 3). These results clearly reveal that males and females continuously experience pronounced stress during reproductive activities that is associated with physiological implications, e.g. elevations in the P/L ratio. When the stress of reproduction has already ended as supported by declining CORT levels during the post-reproductive period, P/L ratios persist on high levels and even continue to augment

in females (Chapter II, Fig. 1). This demonstrates the prolonged immunological effects of chronic stress. However, in both sexes, a marked increase in the stress hormone level occurs shortly before they enter hibernation which suggests that high CORT levels might be necessary for the ultimate strong body mass gain or a physiological switch necessary for entering the long hibernation period. As a result, the post-reproductive reduction in the CORT level in females is short and less pronounced than in males, which may contribute to still rising P/L ratios. Survival probabilities in edible dormice are reduced after high reproductive investment (Lebl et al., 2011; Ruf et al., 2006). The elevated P/L ratio may provide the physiological basis for the reduced survival probability as an indicator of a beginning deprivation of immune function, especially as it sustains for prolonged times after termination of the stressful period. Summed up, our analyses indicate that elevated stress hormone levels enable reproductive success but simultaneously entail physiological impairments on the oxygen delivery and the immune system, which may ultimately contribute to reduced survival.

Effects of hibernation and the post-hibernation status of edible dormice

Variations in physiological parameters further indicate that the post-hibernation period represents a very sensitive and pivotal period for edible dormice and suggest that the pattern of torpor bouts and arousals plays an important role for the physical and physiological condition of an individual upon emergence. Various organs and functions regress during hibernation and require restoration at the beginning of the active season (e.g. Carey et al., 2003). This also includes the restoration of gonads in reproductive years of edible dormice (Schlund et al., 2002). Studies on different hibernating species have shown that cell processes, including mitoses and protein synthesis widely cease during torpor and that especially arousals late in the hibernation period serve for the re-establishment of cellular functions and organs (Carey et al., 2003; Clarke and Fraser, 2004; Kruman, 1992; Kruman et al., 1988), thereby preparing the organism for the subsequent active period. Statistical analyses revealed an effect of the long hibernation period on the RBC picture, the immune cell counts, and stress hormone concentration and suggest an impact of preceding and expected upcoming conditions concerning food availability and reproductive effort. CORT levels were moderately increased at the beginning of the active season (Chapter IV, Fig. 1). For edible dormice, high quality food is still strongly limited at the beginning of the active season while the described restoration processes consume considerable amounts of energy that induce intensified foraging and liberation of energy from internal stores, explaining slightly elevated cortisol concentrations. Erythrocytes seem to be largely unaffected by the long hibernation period. RBC counts and haemoglobin concentration showed a slight increase upon emergence from hibernation compared to levels shortly before hibernation begins, suggesting that erythrocyte production during arousals exceeded erythrocyte death. A high frequency of haemoglobinuria in females indicate a substantial RBC breakdown after

termination of hibernation, indicating that in this sex a substantial amount of senescent erythrocytes accumulated in the course of hibernation but obviously RBC production is sufficient to prevent an anaemic state. In all hibernating species investigated so far, hibernation has been shown to cause a severe leukopenia that applies to all leukocyte subtypes but cell numbers are restored within a few hours after arousal (e.g. Bouma et al., 2010). Interestingly, in these previous studies, the neutrophils in the circulation upon termination of hibernation were predominantly mature (Bouma et al., 2011; Inkovaara and Suomalainen, 1973; Suomalainen and Rosokivi, 1973; Szilagyi and Senturia, 1972). In contrast to these findings, the present study reveals that hibernation in edible dormice results in depleted phagocyte (neutrophils and monocytes) stores that recovered only slowly within the first weeks after hibernation (Havenstein et al., 2016) and those neutrophils detected in the blood were mostly immature or young (band neutrophil). In comparison to other hibernators, dormice hibernate for an extremely long time period of approximately eight months or even longer. Presumably, the short-living neutrophils in dormice completely deplete in the course of the long hibernation period, whereas lymphocytes and erythrocytes with their longer life spans survive this period. A restricted generation of neutrophils seems to occur during late arousals of the hibernation period as a preparative restoration before the start of the active season, which explains the observed pattern of few, young neutrophils. Accordingly, the hibernation pattern and use of late arousals for restoration processes (see e.g.; Carey et al., 2003; Kruman, 1992) presumably determine phagocyte death and renewal. Phagocyte counts were even lower at the beginning of a year of low food availability and reproductive skipping. Hence, edible dormice seem to predict upcoming availability of food and reproductive effort for the subsequent active season (see also Bieber and Ruf, 2009; Fietz et al., 2009; Hoelzl et al., 2015) and adjust the degree of preparative investment into the generation of immune cells during arousals at the end of hibernation accordingly. This contributes to the energy reduction during hibernation, however, it furthermore reflects a trade-off between energy expenditure and immunity. A previous study has shown that mortality rates of edible dormice are highest directly after emergence from hibernation (Lebl et al., 2011). The examined physiological measures provide a functional explanation for this pattern. After termination of hibernation dormice seem to be in a compromised constitution concerning their energetic and immunological state, rendering them vulnerable to infections as well as predation (through intensified foraging activity) which possibly explains the increased mortality rate during this period.

At the beginning of the second of two consecutive high food and reproductive years, CORT levels were higher and erythrocyte parameters of females notify a younger RBC pool (Lutz and Bogdanova, 2013; Willekens et al., 1997) compared to the post-hibernation period of the previous year. Furthermore, the high frequency of haemoglobinuria indicate a heavy erythrocyte breakdown

(Keohane et al., 2015). As explained above, late arousals of the hibernation period are used for the restoration of cells, tissues, and functions (e.g. Carey et al., 2003) and the pattern of the WBC differential suggests that the extend late arousals of the hibernation cycle are used for a preparative restoration of immune cells depends on the expected upcoming reproductive effort and available food resources. RBC haematological data and CORT levels now indicate that females invest stronger into erythrocyte production and renewal during arousals towards the end of hibernation and upon emergence when high quality food was available at the end of the preceding active season and dormice had acquired large energy reserves. It could formerly be shown, that in edible dormice but also in marmots a higher accumulation of body fat reserves results in a different pattern of hibernation characterized by longer arousal periods and shallower torpor bouts, especially towards the end of hibernation (Arnold and Dittami, 1997; Bieber et al., 2014). These findings now suggest a further functional relevance of the different arousal pattern, i.e. there seems to exist a positive carry-over effect of the high food availability on the performance of dormice upon emergence from hibernation, enabling an elevated investment into restoration processes such as into the oxidative transport system. At the same time, the highly frequent haemolytic processes presumably occur due to large proportions of senescent erythrocytes that accumulated in the course of the hibernation period. Females engage strongly in reproductive activities until late in the active season and therefore are assumably unable to perform a comprehensive preparative RBC replacement before entering hibernation which renders an increased RBC turnover necessary after emergence (Chapter III, Table 3, Fig. 3, section "Timing of RBC death and renewal"). As senescent erythrocytes are a source of reactive oxygen species (Belcher et al., 2009), large numbers of elder RBCs might imply elevated oxidative damage in the course of the long hibernation period. Accordingly, besides the positive carry-over effect of high food availability during reproductive years, urinary data concomitantly indicates a negative carry-over effect of reproduction in females.

Effect of food availability

For the ability to cope with demanding situations the availability of food or body energy reserves is an important determinant (Landys et al., 2006). Under food shortage, increased GC levels typically occur due to psycho-nutritional stress and/or the catabolic action of GCs (e.g. Sapolsky et al., 2000). However, in edible dormice no effect of extended food limitation on the stress hormone level and the leukocyte differential could be detected. CORT levels and the P/L ratio do not differ strongly when comparing the period of ripe seeds in a high food year with the respective period of a low food year, proving that food restriction does not cause stress or the need to extensively mobilize fuel from energy stores in edible dormice. On the contrary, CORT concentrations range on basal levels, i.e. CORT levels required for maintaining basic body functions in absence of a demanding challenge.

Studies investigating the metabolic rates of edible dormice clearly showed that edible dormice remain in an "energy saving mode" during extended periods of food limitation, with a greatly reduced oxygen consumption (Bieber and Ruf, 2009; Langer et al., unpublished data). These findings support the hypothesis that edible dormice are able to predict upcoming food availability and reproductive effort and adjust their behaviour, physiology and energetic expenses accordingly (Chapter II, "4.2. Hibernation pattern might determine phagocyte death and renewal", Chapter IV, "Food availability"). On the level of the oxidative system we found significantly reduced RBC counts, haemoglobin concentration and haematocrit under food limitation which is usually interpreted as an impairment in body condition (Huitu et al., 2007; Kalmbach et al., 2004). However, additional RBC indices notify a senescent RBC pool (Lutz and Bogdanova, 2013). A senescent RBC pool is a typical result of a low nutritional status because erythropoiesis is down-regulated under energetic restrictions (e.g. Beldomenico et al., 2008). Therefore, in line with the hypothesis that edible dormice predict upcoming food availability and adjust energetic expenses accordingly, the low values of oxidative capacity measured under restricted food availability are obviously sufficient to meet the physiological needs rather than representing an impairment in body condition. As survival is even increased in a year of low food availability, this reduced erythropoiesis seems to represent part of the energy saving strategy. The results of the physiological measurements demonstrate the ability of dormice to adapt to challenging environmental conditions like extended periods of food limitation.

Study site effects

All physiological parameters indicated least impairments during the demanding reproductive years in the study site with the highest population density (BG), and CORT levels of both sexes were also lowest at this site under low food availability. This consistent pattern clearly indicates a lower stress level and lesser need to cope physiologically in animals from this high density site, irrespective of the different life history stages or environmental conditions. Thus, high population density does not entail elevated adverse effects through competitive encounters for food and shelter. In another study we furthermore found that individuals in BG are larger and heavier than their conspecifics from other sites (Fietz and Weis-Dootz, 2012). This points to the hypothesis that the high population density is rather the result of better environmental conditions and a better performance of edible dormice inhabiting this site than a cause for stress and adverse effects. Consequently, other factors than population density negatively affect individual performance like e.g. predation (Ims and Andreassen, 2000; Sheriff et al., 2009), variations in food availability, logging activities (Bryant and Page, 2005; Meijaard et al., 2005). Among our study sites, BG is the only site that is not regularly logged for economic purposes. Absence of logging may be the ultimate reason for the extremely good performance in terms of stress hormone levels, body masses (Fietz and Weis-Dootz, 2012), P/L

ratios (Havenstein et al., 2016), the oxygen delivery system (Havenstein et al., 2017) and high population density (Fietz and Weis-Dootz, 2012) that we encounter in this study site. However, inter-site variations in these parameters indicate that further study site characteristics like those enumerated above shape individual performance and demographic development, but this deserves further investigation.

Summed up, the CORT concentrations measured in this study sensitively reflect the current level of allostatic load. By combining the results of all physiological measures, the post-hibernation and reproductive periods appear to be the most critical situations for edible dormice whereas prolonged food limitation does not represent a stressful environmental condition. Reproduction imposes physiological costs that manifest in a reduced oxidative capacity, increased stress level, and associated augmentation in the P/L ratio. The latter assumably represents the beginning of stress-induced immunological and health deprivations. Thus, the data support the hypothesis that demanding life history events in dormice require allocation decisions that entail physiological trade-offs which might ultimately trade-off reproduction for survival. Although hibernation is an adaptation to survive unfavorable environmental conditions, the post-hibernation period represents a vulnerable period for dormice. As a result of the long hibernation period dormice are energetically and immunologically in an impaired condition. The need to rehabilitate body functions upon termination of hibernation seems to involve an allocation of resources away from immunity towards restoration processes. This physical state associated with an immunological deprivation may explain the highest probability of mortality during that period of the year in the circannual cycle. Mortality may hit those dormice that already incurred strong physiological impairments during the preceding year, i.e. the period of reproduction or low food availability. In this respect, pre-hibernation fattening and the pattern of hibernation including the usage of arousals for restoration processes is assumed to play a pivotal role for survival after hibernation. These findings prove the viability of physiological parameters for elucidating mechanisms underlying demographic changes. However, the described relationships could only be revealed due to prolonged data acquisition, a detailed analysis of various physiological parameters at the same time and several potentially influencing factors, as well as the in-depth knowledge of the study organism.

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Zusammenfassung

Die verschiedenen Funktionen und Aktivitäten eines Organismus benötigen erhebliche Mengen an Energie und stehen daher im Wettstreit um die nur begrenzt verfügbaren Ressourcen. Daraus resultierende Allokationsentscheidungen zwischen verschiedenen physiologischen Systemen können die Leistungsfähigkeit und die Fitness eines Individuums beeinträchtigen. Die Untersuchung dieser physiologischen *„Trade-offs*“ kann Aufschluss über die Mechanismen geben, die der Evolution von *„Life History“* Strategien und Populationsrückgängen zugrunde liegen. Da endokrine Mediatoren, insbesondere Glucocorticoid Hormone (GCs), die physiologischen Reaktionen eines Organismus auf Stimuli aus der Umwelt vermitteln und diverse Körperfunktionen regulieren, sind Variationen in GC Konzentrationen von großem Interesse für ökophysiologische Studien. Das Immun- und das Sauerstofftransportsystem sind für das Überleben essentielle Körperfunktionen, die sensitiv auf veränderte Umweltbedingungen, Nähstoffmangel sowie auf Stresshormonanstiege reagieren. Dementsprechend sind weiße und rote Blutzell- (WBC und RBC) Indizes geeignet, den Gesundheitszustand und die körperliche Verfassung von Individuen zu beurteilen und stellen somit weitere wichtige Erhebungsparameter in ökophysiologischen Studien dar.

Unser Studientier, der Siebenschläfer (*Glis glis*), ist ein arboreales Nagetier aus der Familie der Bilche (Gliridae) mit einer Körpermasse von ungefähr 100 g. Er zeichnet sich durch eine außerordentlich lange Winterschlafdauer von etwa acht Monaten und einer hohen Synchronisierung im Jahreszyklus aus. Ziel dieser Studie war, anhand von Variationen in WBC und RBC Parametern, Urin-GC Konzentrationen sowie dem Auftreten von Hämoglobinurie die Auswirkungen von anspruchsvollen Situationen wie Winterschlaf, Reproduktion, begrenzte Nahrungsverfügbarkeit und hohe Populationsdichte zu untersuchen und Rückschlüsse auf die physiologischen Mechanismen zu ziehen, die der Evolution von "*Life History“* Strategien und Populationsdynamiken zugrunde liegen. Dafür wurden freilebende Siebenschläfer beider Geschlechter von 2012 - 2014 in ihrer aktiven Phase in fünf verschiedenen Untersuchungsgebieten im Südwesten Deutschlands untersucht.

Ergebnisse zeigen, dass die ersten Wochen nach dem Winterschlaf eine äußerst sensible Phase für Siebenschläfer darstellt, da die phagozytierenden Zellen, ihre immunologisch erste Verteidigungslinie, offensichtlich über die ausgedehnte Winterschlafzeit absterben und sich deren Zahlen zu Beginn der aktiven Saison nur langsam erholen. Die Notwendigkeit, nach dem Winterschlaf in die Regeneration zurückgebildeter Organe zu investieren, während qualitativ hochwertige Nahrung noch fehlt, führt offensichtlich zu leicht erhöhten GC-Leveln, um Energie für diese Wiederherstellungsprozesse zu mobilisieren. Zu Beginn eines Jahres mit geringem Futterangebot ist das Phänomen der erniedrigten Phagozytenzahl sogar noch verstärkt und die wenigen im Blut vorhandenen Neutrophilen sind jung oder unreif. Dies deutet darauf hin, dass Siebenschläfer in der Lage sind, die

bevorstehende Futterverfügbarkeit und den Reproduktionsaufwand vorherzusehen und dass sie bereits gegen Ende der Winterschlafphase ihre Zellprozesse, angepasst an die erwarteten Verhältnisse, aktivieren. Jedoch scheint die Rehabilitation der Zellen des angeborenen Immunsystems aufgrund des negativen Energiestatus nach dem Winterschlaf eingeschränkt zu sein. Während dieser Zeit im Jahreszyklus der Siebenschläfer ist ihre Überlebenswahrscheinlichkeit am geringsten, demzufolge zieht dieser „*Trade-off*" zwischen Energieaufwand und Immunität nachteilige Konsequenzen für die Fitness der Tiere nach sich.

Erhöhte Cortisol-Konzentrationen während der Reproduktion zeigen, dass dieses „*Life History*" Ereignis Männchen wie Weibchen stark beansprucht. Gleichzeitig tritt eine Beeinträchtigung des Sauerstofftransportsystems sowie ein deutlicher Anstieg im Verhältnis der Phagozyten zu den Lymphozyten Zahlen (P/L Quotient) auf, welches eine Stressantwort des Immunsystems darstellt. Die Verminderung der Sauerstofftransportkapazität scheint größtenteils auf energetische Limitierungen und einen Mangel an Nährstoffen bei gleichzeitig gehäuft auftretenden alternden Erythrozyten zurückzuführen zu sein. Die insbesondere bei Weibchen häufig auftretende Hämoglobinurie unterstützt diese These. Nach Beenden der Reproduktionsaktivitäten gehen die Cortisolkonzentrationen zurück, während die massiven Anstiege im P/L Quotient bis zum Ende der aktiven Saison bestehen bleiben, was auf nachhaltige Auswirkungen von chronischem Stress auf das Immunsystem hindeutet. Da die Mortalität in Reproduktionsjahren bei Siebenschläfern deutlich erhöht ist, weisen die hohen Cortisolwerte während der Reproduktion auf eine allostatische Überlastung hin. Der hohe P/L Quotient scheint in diesem Zusammenhang einen entscheidenden Indikator für eine beginnende Verminderung der Immunfunktion durch chronischen Stress darzustellen.

Das Ausbleiben eines Anstiegs der Cortisolkonzentration und des P/L Quotienten bei lang anhaltender Futterlimitierung stützt die Hypothese, dass die zukünftige Futterverfügbarkeit für Siebenschläfer vorhersehbar ist und zeigt, dass ein Mangel an hochwertiger Nahrung, unter anderem aufgrund der Vorhersehbarkeit, keinen Stress verursacht. Im Spätsommer eines Futtermangeljahrs ist die Sauerstofftransport-Kapazität reduziert, was üblicherweise als Beeinträchtigung des allgemeinen Körperzustands interpretiert wird. Allerdings zeigen weitere RBC-Parameter einen alternden Erythrozyten-Bestand an. Dies lässt darauf schließen, dass eine reduzierte Erythrozytenproduktion Teil einer Energie-Einspar-Strategie ist. Da die Überlebenswahrscheinlichkeit in Jahren begrenzter Futterverfügbarkeit hoch ist, demonstrieren diese Ergebnisse die Anpassungsfähigkeit von Siebenschläfern an eine lang andauernde Limitierung in der Nahrungsverfügbarkeit.

Diese Studie zeigt, dass Urin-Cortisolkonzentrationen präzise die hormonelle Antwort der Siebenschläfer an unterschiedliche Herausforderungen abbilden. Hämatologische Parameter stellen verlässliche Indikatoren für die Beurteilung der physiologischen Auswirkungen dar, mit dem Potential, physiologische und Fitness "Trade-offs" aufzudecken. Die breit aufgestellte Analyse

verschiedener Parameter, ermöglicht die Abgrenzung der Einflüsse verschiedener Stressoren und ein ganzheitliches Verständnis der komplexen Zusammenhänge unter natürlichen Bedingungen.

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