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Overwintering Strategies of a Boreal Small Mammal in a Changing Climate

JYVÄSKYLÄN YLIOPISTO

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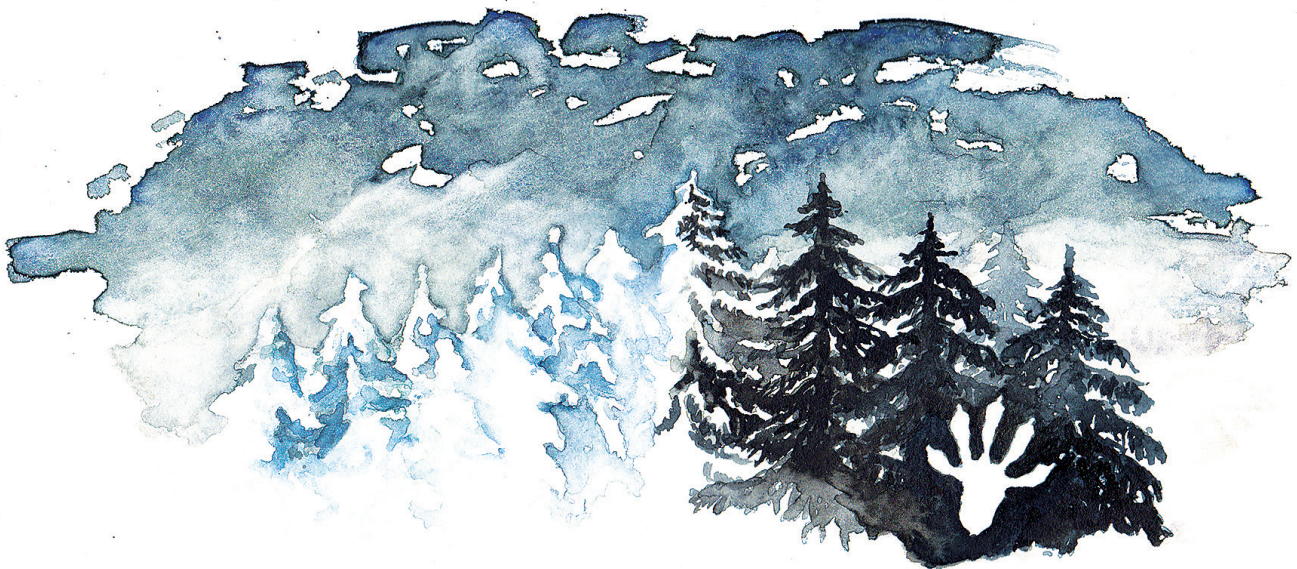
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ABSTRACT

The global climate change is predicted to cause notable alterations in winter conditions in northern areas. This raises many questions about its possible consequences on northern species and ecosystems. In many areas the time period with an intact snow cover is about to get shorter or even vanish altogether. As the overwintering success of many small mammals is strongly dependent on the protection provided by the snow cover against severe weather conditions and predation, it is obvious that these kinds of environmental alterations should affect the overwintering physiology and behavior, as well as the initiation of breeding after winter. In this thesis I have studied the winter ecology of a small boreal rodent species, the bank vole (*Myodes glareolus*), and the possible consequences of climate change on the physiology, behavior and onset of reproduction in this species. I conducted four experiments under laboratory (II-IV) and semi-natural conditions (I, III). One of my experiments was purely methodological (IV), providing me with validated and accurate endocrinological study methods, and thereby enabling my other experiments. I found that the sex-ratio of the overwintering population affects the physiology and behavior of bank voles and thus, leads to divergent overwintering strategies (I). I was also able to show that the changing winter conditions related to climate change alter the circadian activity pattern and possibly even the anti-predator behavior of this species (II). Further, my studies showed contrasting sex differences in the response to environmental cues regulating the onset of reproduction after winter (III). In case of rapid environmental changes, this could lead to a reproductive mismatch between the sexes and thus, strongly affect fitness. The results of my thesis provide new information about the overwintering mechanisms of a winter-active small boreal rodent, and may help us to predict some of the possible consequences of climate change on northern boreal ecosystems.

Keywords: Climate change; overwintering; onset of reproduction; physiology; behavior, predation risk.

Saana Sipari, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address Saana Sipari
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
saana.m.sipari@jyu.fi

Supervisors Prof. Hannu Ylönen
Department of Biological and Environmental Science
Konnevesi Research Station
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Docent Ines Klemme
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Docent Janne Sundell
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers Prof. John S. Millar
Department of Biology
University of Western Ontario
London
ON N6A 5B7
Canada

Prof. Anders Angerbjörn
Department of Zoology
Stockholm University
S-106 91 Stockholm, Sweden

Opponent Prof. Rudy Boonstra
University of Toronto
Scarborough
1265 Military Trail
Toronto, ON M1C 1A4
Canada

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

I   Sipari, S., Haapakoski, M., Klemme, I., Palme, R., Sundell, J., Ylönen, H. Population sex-ratio affecting behavior and physiology of overwintering bank voles (*Myodes glareolus*). Manuscript.

II   Sipari, S., Haapakoski, M., Klemme, I., Palme, R., Sundell, J., Ylönen, H. Changing winter conditions in the boreal forest: The effects of fluctuating temperature and predation risk on activity and stress in bank voles. Manuscript.

III   Sipari, S., Haapakoski, M., Klemme, I., Sundell, J., Ylönen, H. 2014. Sex specific variation in the onset of reproduction and reproductive trade-offs in a boreal small mammal. *Ecology* 95: 2851-2859.

IV   Sipari, S., Palme, R., Ylönen, H. Validation of enzyme immunoassays and evaluation of factors affecting the levels of fecal steroid metabolites in bank voles (*Myodes glareolus*). Manuscript.

The table shows the contributions of the authors to the original papers.

	I	II	III	IV
Original idea	HY	SS, HY	SS, HY	SS, HY, RP
Experimental design	SS, HY, MH, IK,JS	SS, HY, MH, IK, JS	SS, HY, MH, IK, JS	SS, RP
Data collection	SS, MH	SS	SS	SS
Data analyses	SS, MH, RP	SS, RP	SS, IK	SS, RP
Writing*	SS	SS	SS	SS

SS = Saana Sipari, HY = Hannu Ylönen, MH = Marko Haapakoski, IK = Ines Klemme, JS = Janne Sundell, RP = Rupert Palme

Writing*: I was the main writer in all articles (I-IV), but all authors named in the respective studies contributed to the writing with several comments and notations.

1 INTRODUCTION

Seasonality plays an essential role in the life histories of many species, affecting their physiology, behavior, reproduction and survival. The strongest seasonality occurs in high latitude areas, like the Arctic and boreal regions, where spring, summer, autumn and winter all represent distinct periods in the annual cycle. In these areas winter, the longest of the four seasons, is often the single most influential season for the life history of most of the organisms, especially vertebrates (Marchand 1996). Successful overwintering is not solely a matter of survival for the individual but it can also determine the fate of the following breeding season. Thus, understanding the determinants of successful overwintering provides important information on the ecology of a species, as well as insights into causes and effects of many trophic interactions in northern ecosystems.

Various physiological and behavioral mechanisms have evolved to enable animals to cope with the harsh environmental conditions of the northern winter. For homeothermal winter-active species, like many boreal mammals, the neuroendocrine system called hypothalamic-pituitary-adrenal axis (HPA axis = stress axis) is essential in equipping the animals to endure challenging winter conditions (Boonstra 2004, Smith and Vale 2006, Boonstra 2013, Boonstra *et al.* 2014). Winter adaptations such as accumulation of fat reserves, thick winter pelage and adjusted energy metabolism are all derived by the HPA axis. It also regulates stress reactions and reproductive status, as well as the winter behavior of overwintering animals (Boonstra *et al.* 2014). Nevertheless, many of the winter-active animals could not survive without the most characteristic feature of the northern winter, snow (Marchand 1996). Snow cover protects the ground from freezing and provides shelter for many small ground-dwelling animals, such as rodents and shrews against cold and predators. In the subnivean space formed between the ground and the snow pack, temperature stays constantly around 0^0C despite the strongly fluctuating ambient temperature. Many different species exploit the subnivean space, some for foraging, some for insulation and nesting, and some for hiding. In many

species, overwintering strategies are partly or entirely based on exploiting the snow cover (Marchand 1996).

Thus, it is understandable that the recent course of global climate change has raised many questions about its possible consequences on northern ecosystems, as notable alterations in winter conditions are predicted to occur (Serreze *et al.* 2000, Moritz *et al.* 2002, Rasmus *et al.* 2004, Lovejoy and Hannah 2005, Jylhä *et al.* 2008, Stocker *et al.* 2013). Scenarios drawn by the IPCC (Intergovernmental Panel for Climate Change) suggest that in many areas the time period with an intact snow cover will get shorter or vanishes altogether. The autumn will become longer and spring will arrive earlier. Also, the unstableness and unpredictable events in climate will be more pronounced than before (Lovejoy and Hannah 2005). The winter precipitation is estimated to increase but due to the less frequent frost days, it may more often come down as rain, melting the snow pack partly or even entirely (Jylhä *et al.* 2008, Jylhä *et al.* 2009). This would leave the ground exposed to freezing, and could cause formation of an ice layer on top of the ground, which could notably deteriorate the foraging possibilities for many animals (Putkonen and Roe 2003, Stien *et al.* 2012). Also, the quality of snow is likely to change due to the more frequent rain-on-snow events, which could alter its insulation value (Rasmus *et al.* 2004, Jylhä *et al.* 2008).

For small mammals, the main threats in winter are low temperature, predators and starvation (Marchand 1996, Boonstra *et al.* 2014). In the future, the less reliable snow conditions, and in the worst case the total lack of the snow cover, could notably intensify the impact of these stressors. Under unfavorable external conditions animals face trade-offs, and they often allocate resources from less vital functions to survive. However, under prolonged effects of the stressor or in case of multiple simultaneous stressors, as described above, the reallocation of resources gets more demanding and can eventually lead to severe neglect and deterioration of many physiological functions (McEwen and Wingfield 2003, McEwen 2004, McNamara and Buchanan 2005, Beldomenico *et al.* 2008, Bronson 2009). Ultimately, this may result in significantly decreased over-winter survival.

The experienced winter conditions can also have further consequences, as they can affect the initiation and success of the oncoming breeding season. In addition, the advanced snowmelt and onset of spring due to the climate change is suggested to cause serious mis-matches and erroneous timings in ecological events, like reproduction in animals and sprouting in plants. With some species, particularly in birds, there are already indications of such events (Visser *et al.* 1998, Both and Visser 2001, Stenseth and Mysterud 2002, Berteaux *et al.* 2004, Parmesan 2006, Ludwig *et al.* 2006, Visser *et al.* 2009). Besides trophic mismatches, there may also be sex-specific reactions to the changing winter and spring conditions, because of different selection pressures on females and males (Trivers 1972, Ball and Ketterson 2008, Sheriff *et al.* 2013). If so, this could noticeably decrease the fitness of the individuals in many seasonally breeding species.

Adapting to these rapidly changing environmental conditions requires both physiological and behavioral plasticity. This emphasizes the importance of the functions of the neuroendocrine system, as it is considered to be one of the main factors defining the fate of many species living under changing boreal climate conditions (Boonstra 2013; Wingfield 2013; Boonstra et al. 2014).

This thesis is comprised of four individual studies, using a common boreal winter-active rodent, the bank vole (*Myodes glareolus*), as a model species. First, I studied the behavior and social dynamics in overwintering small mammal populations in order to determine the overwintering strategies under current winter conditions. I assessed the role of sex, social interactions and sex ratio of the population on early winter behavior and survival of this species (I). Second, in order to test my hypothesis that the climate change should induce elevated stress levels in overwintering small mammals, I performed a laboratory experiment mimicking late winter temperature fluctuations and predatory cues as stressors that simulated the exposure to unstable temperature and increased predation pressure caused by the lack of a snow cover (II). Third, in a combined laboratory and field enclosure experiment I examined how two different environmental cues, variability of temperature and nutritional conditions in spring, affect the sex-specific onset of breeding in bank voles (III). Fourth, to provide me with validated and accurate endocrinological study methods, I performed a validation experiment for two enzyme immunoassays (EIA) for measuring fecal steroid metabolites, as an indication for hormonal status in bank voles. I also assessed the factors affecting the excreted metabolite levels, such as sex, time of the day and excretion route. The validated methods and EIAs were used in experiments I and II.

My objective in this thesis is to provide new information on overwintering strategies in bank voles, as well as assessing the possible consequences of the changing climate on winter time survival, behavior and the initiation of breeding in this winter-active boreal small mammal.

2   METHODS

2.1   Study species

The bank vole is a common winter-active small rodent which is distributed over a wide geographical range in the Palearctic (MacDonald 2001). It is an omnivorous species with a diet including seeds, plant parts, fruits, mosses, lichens, fungi and invertebrates (Butet and Delettre 2011). The protein content of its food is suggested to be important for maturation and reproductive success, especially for females (Von Blanckenhagen *et al.* 2007). In boreal latitudes, the breeding season is relatively short and usually occurs from April-May to September (Kaikusalo 1972, Koivula *et al.* 2003). Bank voles have a promiscuous mating system (Klemme *et al.* 2006), and females often mate postpartum, i.e. shortly after delivery, allowing them to produce several litters during short period of time (Gustafsson *et al.* 1980). During the breeding season females defend their exclusive breeding territories, whereas the home ranges of males are larger and overlap with each other and with several female territories (Bujalska 1973). However, it is suggested that during winter their social tolerance increases and similar to many *Microtus* species they exploit communal winter nests for more efficient thermoregulation (Ylönen and Viitala 1985, Ylönen and Viitala 1991). Under stress the main glucocorticoid released into the blood in bank voles is corticosterone (Boonstra 2004).

The experimental animals used for these studies were wild captured individuals trapped in Konnevesi, Central Finland (62°37′N, 26°20′E), or individuals from the laboratory colony, maintained at Konnevesi research station. All animals were marked with ear tags for individual identification.

2.2 Overview of study designs

2.2.1 Experimental procedures

A simplified overview of the experimental setups is presented in Table 1.

All experiments were carried out in the laboratory at the Konnevesi Research Station and/or in large (0.25 ha) outdoor enclosures near Konnevesi municipality. For experiments II and III, I used temperature adjustable climate chambers and for experiment IV a standard animal laboratory. During the laboratory experiments voles were housed individually in standard mouse cages (43×26×15 cm). For the field enclosure study (I) and combined laboratory -enclosure study (III) I used outdoor enclosures. The enclosures were made of galvanized steel sheet of 125 cm of height, with the fence reaching 0.5 m under the ground and 0.75 m above ground. The fence prevents voles from escaping and small mammalian predators from entering the study area. The enclosures do not prevent avian predation. The habitat was homogenous old meadow with mainly tall grass and bushes as vegetation. Twenty five Ugglan multiple capture live traps (Ugglan special, Grahnab ab, Hillerstorp, Sweden) distributed evenly in a 5 x 5 grid in each enclosure allowed the monitoring of the experimental populations. All traps were covered with trap chimneys (40 x 40 x 50 cm) made of metal sheet, which allowed trapping during the snowy season (I). Sunflower seeds were used as bait.

In study I, I manipulated population sex ratios to be either female biased, male biased or even. In studies II and III I manipulated the diel temperature regime, exposing animals either to stable or unstable temperature conditions. Additionally, in study II, the animals were exposed to predator sound or control sound, whereas in study III the food quality was manipulated. In study IV I manipulated the steroid levels of the test animals by adrenocorticotropic hormone (ACTH) injections and radiolabelled corticosterone and testosterone injections.

Survival (I, III) and space use (I) of the animals in field studies were monitored by live-trapping. The space use data was analyzed with Ranges 6 program (Anatrack ltd, Wareham, UK), which calculated the movement areas of the animals, as well as the degree of overlapping areas between individuals. The degree of overlapping movement areas were used as an indicator of winter aggregation and social tolerance in overwintering voles (I). Corticosterone metabolite levels were measured using fecal samples in studies I, II and IV. Fecal testosterone metabolites were measured in study I and IV. In study III testosterone was monitored using blood samples. Food consumption was measured by weighing the food given to the animals and weighing the leftovers (II, III). Body mass was measured using an electronic balance (I, II, III, IV) or Pesola spring balance (I).To monitor the activity in study II, the animals were injected with subcutaneous PIT tags, and their cages were equipped with a sensor (Trovan®, EID Aalten BV, Aalten, Holland) connected to a PC, which

recorded their movements. Breeding condition of the animals was estimated by monitoring testosterone levels in males and vaginal opening in females (III).

2.2.2 Endocrine monitoring

In study IV, I validated two enzyme immunoassays (EIA) for measuring fecal steroid metabolites in bank voles. These EIAs were used for monitoring the hormonal status of the animals in studies I and II.

Fecal samples were collected both under laboratory conditions (I, II) and in the field (I). To collect the samples under laboratory conditions each vole was temporarily moved to an empty (no beddings) mouse cage (43×26×15 cm). After the vole defecated, it was returned to its home cage. This usually happened fast, and took max. one hour. After this, feces were collected to empty Eppendorf-tubes (1.5 ml, one tube per individual) from the sampling cage using tweezers. In the field, the samples were collected from the floor of the traps and the traps were wiped clean with paper towels after each sampling. The traps were checked every three hours, three times per day. Samples were stored at -20 ˚C. To measure corticosterone metabolite (stress) levels, the samples were analyzed by using 5α-pregnane-3β,11β,21-triol-20-one EIA with methods described in Touma et al. 2003. For testosterone metabolite measures the 17ß-hydroxyandrogen EIA was used. All laboratory analyses were performed at the University of Veterinary Medicine, in Vienna, Austria.

To measure testosterone in study III, I used blood samples collected with retro-orbital bleeding. Blood samples were analysed by using a commercial radioimmuno assay (TESTO-CTK, DiaSorin, Byk-Sangtec Diagonstica GmbH & Co, Germany). Laboratory analyses were performed at the University of Jyväskylä, Finland.

TABLE 1   Overview of the experimental setups in the studies (I-IV) including place, subject of interest, manipulations and the measured variables in each study.

	I	II	III	IV
Place	enclosure	climate chamber	climate chamber enclosure	laboratory
Subject	overwintering	multiple stressors	onset of breeding	assay validation
Manipulation	sex-ratio	temperature predation risk	temperature food quality	ACTH challenge radiometabolism
Measured variables	survival space use fecal corticosterone metabolite levels (stress) fecal testosterone metabolite levels body mass	fecal corticosterone metabolite levels (stress) activity food consumption body mass	breeding condition (testosterone in males, vaginal opening in females) onset of breeding food consumption body mass	excretion of corticosterone and testosterone metabolites

3 RESULTS AND DISCUSSION

3.1 Overwintering under current winter conditions

Many boreal rodent species that are solitary and territorial during the breeding season are known to become more social during winter and construct communal winter nests for more efficient thermoregulation (Webster and Brooks 1981, West and Dublin 1984, Ylönen and Viitala 1985, Ylönen and Viitala 1991, Merritt and Zegers 2002, but see Berteaux *et al.* 1996). However, the degree of communal nesting seems to vary between species, individuals, years, habitats and winter characteristics (Viitala 1984, West and Dublin 1984) leading to an incomplete understanding of this winter behavior. Here, I suggest that the sex-ratio of the overwintering population may affect the overwintering strategies in bank voles (I). We observed that the tendency for winter aggregation and presumed communal nesting was notably lower in male biased populations than in female biased or even sex-ratio populations. Also, stress levels measured as fecal corticosterone metabolite concentrations were highest in male biased populations. The elevated stress levels and the tendency to avoid overlapping with movement areas of other individuals could indicate a solitary overwintering strategy and antagonistic social dynamics between individuals. This supports the suggested role of females as the initiators and maintainers of winter aggregations and communal nests. Another possible explanation for the solitary behavior could be the larger body size and thus, higher absolute energy need of males. In a population with high male density and limited food resources, this could lead to strictly defended territories to ensure sufficient food supply for the winter (West and Dublin 1984, Dantzer *et al.* 2012). The higher stress levels observed in male biased populations could originate from territorial conflicts as well as cold stress due to the possible solitary nesting. Interestingly, the suggested antagonistic behavior in males living in male biased populations appears not to be testosterone induced. Thus, we suggest that, similar to e.g. red squirrels (Boonstra *et al.* 2008, Dantzer *et al.* 2012), the winter aggression in bank vole males could be related to the hormone dehydroepiandrosterone (DHEA) (Boonstra *et al.* 2008). This, however, needs to be verified.

A female biased sex-ratio in the overwintering population resulted in notably different behavior. I found strong indications of aggregation and possibly communal nesting. Further, the higher female density in the populations induced significantly elevated testosterone metabolite concentrations in males, and winter breeding was observed. In even sex-ratio populations there was also an indication of winter aggregation but no significant hormonal responses were observed. During the early winter these different strategies did not result in differences in survival. Also, no significant differences in survival between the sexes were observed. However, irrespective of the population type, the autumn body mass affected the survival probability in winter. It is known that small rodents decrease their body mass before winter to reduce energy requirements (Hyvärinen 1984, Marchand 1996, Aars and Ims 2002). Yet, my results show that females with the lowest body mass in the autumn survived significantly worse than heavier individuals. Thus, the trade-off between decreased energy requirement due to smaller body mass, and increased energy consumption via heat loss due to the increased surface-area-to-volume ratio needs to be adjusted carefully for an optimal balance.

3.2 Overwintering under changing winter conditions; speculations and suggested scenarios

In the future, under unreliable snow conditions or total lack of snow, the divergent overwintering strategies observed in study I, could lead to notably different outcomes in survival, although not observed in here. Without the insulating snow cover the role of social thermoregulation, i.e. communal nesting, would be highly emphasized. Even under the predicted warmer winter conditions, temperatures are still low and extreme weather conditions possible. Thus, females as initiators and maintainers of communal nests could play an essential role in the overwintering survival of future populations. On the other hand, the more frequent rain-on-snow events or lack of snow cover could lead to the formation of an ice layer on the ground and notably hinder foraging. Under limited food availability the trade-off between saved energy due to social thermoregulation, and lower energy gain due to the decreased food availability per individual, should be carefully evaluated. However, winter survival is the sum of many factors. For instance, in case of solitary overwintering, the elevated corticosterone levels we observed in male biased populations, likely derived from territorial conflicts and cold stress, could in the long run lead to declined body condition and lower survival, despite the possibly higher food gain compared to the social overwintering strategy. For males, who are typically larger than females and have higher energy needs, combined with the assumption that their possibilities for communal nesting could be dependent on females, the future scenario is challenging. Whether the high density of females in overwintering populations will also under future conditions induce winter

breeding, like observed in our study (I), is difficult to estimate. The energetically demanding winter breeding could easily result in high mortality of both the pups and nursing females in case of sudden deterioration in weather or food conditions (Millar 2007). Also, maintaining high testosterone levels to enable breeding negatively affects survival in males. Nevertheless, due to the opportunistic nature of small short lived animals, it is likely that the winter breeding may occur occasionally despite the challenging changes in winter conditions.

In the case where one of these overwintering strategies, solitary or social, would become favored over the other due to the changes in winter conditions, alterations in the selection pressure for body mass or the ability to adjust the body mass could occur. As observed in study I, the balance between energy requirement and the rate of heat loss should be adjusted carefully. Considering the solitary overwintering strategy, larger individuals should be favored over smaller ones. Larger body size would decrease the relative surface area and thus lower the heat loss. Without the benefits of social thermoregulation and insulation of snow cover this would be essential. On the other hand, if the social overwintering strategy and communal nesting would be favored, the opposite trend in body mass would apply. Social thermoregulation could permit smaller body size, which in turn would be beneficial under limited food resources. However, as the females in study I showed, there is a limit when small becomes too small, and compromises the survival regardless of the chosen strategy.

Besides the speculated effects on overwintering strategies (solitary or social) our results suggest that the predicted changes in winter conditions could also affect the circadian rhythm and even the anti-predator behavior of this species (II). During late winter and early spring, with increasing day length, the differences in day and night temperatures become more prominent. During the day, temperature can rise above zero whereas during the night it may drop even twenty degrees (°C). The snow cover dampens these differences to non-existent, but without it, ground-dwelling animals are exposed to radical temperature fluctuations. In study II, when exposing the animals to fluctuating diel temperature regime, with cold nights and warmer days, we observed that their normally more nocturnal polyphasic activity pattern changed towards a more diurnal rhythm. Surprisingly, being exposed to fluctuating temperatures did not cause elevated stress levels measured as corticosterone metabolites. Thus, it appears that bank voles are able to adjust their circadian activity patterns according to their environment, and so avoid or diminish the physiological effects of the stressor.

To test whether simultaneous exposure to multiple stressors would lead to an accumulation of negative stress effects, I added predatory cues along the temperature manipulation. Animals were exposed either to owl calls or to the sound of a non-predatory bird species. Introducing the sound manipulation elevated the stress levels in females but not in males. Also, the laboratory born voles were not able to distinguish between the sound of non-predatory control bird and predatory bird, but seemingly considered both as a threat. Introducing the sound manipulation caused no differences in stress levels between

individuals experiencing either unstable or stable temperatures. Thus, unlike expected, the exposure to two assumed stressors at the same time did not cause higher stress levels or an accumulation of stress effects, e.g. in form of reduced body mass. Despite the lack of a stress response, the temperature treatments seemed to affect the behavioral response when encountering a threatening sound. Voles under unstable temperature reduced their overall activity when exposed to the predator sound, whereas under stable temperature this reaction was not observed. This could indicate that abiotic environmental conditions may affect the anti-predator behavior in bank voles.

Anti-predator behavior is highly dependent on multiple factors (Ydenberg and Dill 1986, Eilam *et al.* 1999, Edut and Eilam 2004, Stankowich and Blumstein 2005, Blumstein 2006, Cooper *et al.* 2012) and one should be careful with the conclusions drawn from laboratory experiments. Still, when speculating on the feasibility of these observed responses under possible future winter conditions, one could argue that the reduced activity under predation risk may be beneficial. Without the visual protection provided by the snow cover or dense vegetation, the reduction of mobility could be the most favorable anti-predator response. But in the long run this could lead to energy deficiencies if the food availability is scarce, as well as to an accumulation of scent cues for predators (Banks *et al.* 2000, Ylönen *et al.* 2003, McNamara and Buchanan 2005). The latter applies especially to the social overwintering strategy, where several animals are clustered in the same spot. The shift toward more diurnal circadian rhythm observed under the unstable temperature treatment would be a good behavioral adaptation against nocturnal predators and cold nights, but on the other hand, it could increase the likelihood of being preyed upon by day active predators.

3.3   Onset of reproduction after winter

In seasonal environments, the breeding season for many species is relatively short. Thus, it is important to time the onset of reproduction in a way that maximizes the reproductive output of the individual. Starting too early can lead to nest failure, causing energy and time loss, whereas starting late shortens the potential time for reproduction and possibly reduces the number of available nesting sites or territories (Millar and Gyug 1981, Sharpe and Millar 1991). To assess the optimal time for the onset of reproduction, animals follow cues from the surrounding environment, such as photoperiod, temperature and abundance of food (Gwinner 1986, Bronson 1989, Hahn *et al.* 1997, Futuyma 2005, Karasov and del Rio 2007) In study III, I tested the hypothesis that females and males respond differentially to environmental stimuli due to the different selection pressures they face (Ball and Ketterson 2008, Sheriff *et al.* 2013) by manipulating food quality and temperature at the onset of breeding. The results showed that male maturation was advanced under variable temperature conditions compared to stable temperature conditions. Perhaps, for northern

rodents the strongly fluctuating temperature could indicate the snowmelt and onset of spring (Spencer 1984). Female maturation, on the other hand, occurred significantly earlier under stable temperature conditions. This may be explained by the higher costs of reproduction that females face compared to males (Trivers 1972). Unexpectedly, the quality of food, high or low protein, did not affect the onset of breeding in this study. I also observed that unstable climatic conditions experienced during maturation in early spring can have a negative delayed effect on survival later in the breeding season.

Under changing environmental conditions sex differences in the response to environmental cues, as observed in our study, may lead to a reproductive mismatch between the sexes. If the melting snow cover in the spring induces maturation and breeding condition in males (Spencer 1984), it suggests that in the future male maturation could be clearly advanced. However, if the females will delay their maturation due to the unstable and more unpredictable weather conditions, it causes a serious asynchrony between the sexes. Early maturation in males can provide a competitive advantage in case they can locate receptive females, but without the possibility for breeding maintaining the high testosterone level is serious waste of energy and can also cause immunosuppression (Mills *et al.* 2009, Boonstra *et al.* 2014). This can negatively affect survival. Yet, the later onset of winter could enable the breeding season to continue longer in the autumn if decreased day length does not limit the length of the breeding season. Due to the different life history strategies in males and females, the sex differences in cues triggering the reproduction could be relatively common among animals. Thus, the rapidly changing environmental conditions could notably decrease the fitness of many seasonally breeding species.

3.4 Physiology and behavior; monitoring the hormonal status

The neuroendocrine system regulates all essential bodily functions and plays an important role adapting animals to their environment (Boonstra 2004, Campbell and Reece 2005, Smith and Vale 2006, Boonstra *et al.* 2014). Thus, monitoring the hormonal status of an animal can help explaining an observed behavior or body condition. However, endocrinological studies can be problematic, as the sampling procedure itself can significantly alter the hormonal levels or the behavior of the animal, leading to incorrect conclusions (Gartner *et al.* 1980, Haemisch *et al.* 1999). This is often the case e.g. with blood sampling used for monitoring hormones with fast reaction to stimuli, such as glucocorticoids. To avoid these problems, using feces for endocrine monitoring has lately become increasingly popular. However, several factors need to be taken into account, such as the species-specific circadian fluctuation of particular hormones, gut passage time, excretion route and sex specific differences. Also, the original hormone is rarely present in feces, and hormone metabolites are often the only measurable content available in the excreta (Palme and Möstl 1997, Touma *et al.*

2003). As the types of metabolites can vary between species and even sexes, it is important to validate the chosen assay to be sure that it is suitable for the purpose. In study IV, I validated two enzyme immunoassays for measuring fecal corticosterone and testosterone metabolite levels in bank voles, and evaluated the factors affecting the levels of these steroid metabolites. These particular steroids were chosen for their significant role in behavioral and physiological functions in bank voles. The results show that there is a significant circadian fluctuation in the secretion of both corticosterone and testosterone. Males had significantly higher corticosterone metabolite levels than females and their main excretion route was via feces, whereas in females the ratio between feces and urine was nearly half-and-half. This is important to take into account when comparing measured stress levels between the sexes. The gut passage time in corticosterone metabolites was 6-8 hours, similar in both sexes. However, there was a strong variation between individuals in the excretion rate, especially in males, which can be disturbing in case of small sample sizes. Naturally, the testosterone metabolite levels were significantly higher in males than in females. In testosterone the main excretion route was via feces both in males and females, and the gut passage time was around 16 hours. These validated enzyme immunoassay were used in studies I and II.

The functions of the neuroendocrine system and the plasticity in the responses of animals are considered to be the main factors defining the fate of different species under the changing climate conditions (Wingfield and Hunt 2002, Wingfield 2013, Boonstra *et al.* 2014). Thus, providing a validated and practical method for monitoring these variables is of high relevance when trying to understand the ecological as well as evolutionary consequences of the climate change for different species.

4 CONCLUSIONS

The ultimate impacts of climate change will be highly intricate as the predicted changes affect both abiotic and biotic conditions and their spatial and temporal interactions in the ecosystems. These global and local alterations have already caused changes in trophic interactions and distribution of species. Thus, it is impossible to appraise all the possible consequences of the changing climate conditions for the life history of a particular species. However, I believe that an experimental approach is a valuable method for answering some of these questions.

To assess the potential effects of the changing climate conditions on the overwintering of a species is not possible without knowledge of its winter ecology under the current environmental circumstances. Thus, I performed a study examining the behavior and social dynamics in the overwintering populations under semi-natural conditions (I). In this study I was able to show that the sex-ratio of the overwintering population may have a strong effect on the winter time behavior and physiology of an individual, leading to divergent overwintering strategies between populations. Further, the results from the study II suggest that the future changes in winter conditions due to climate change may alter the circadian activity pattern and possibly even the anti-predator behavior in bank voles. I was also able to show clear sex differences in the response to environmental cues regulating the onset of reproduction after winter (III). As the hormonal monitoring provides valuable information about the animal's physiological and behavioral responses to environmental conditions, I validated two enzyme immunoassays for measuring fecal corticosterone and testosterone metabolite levels in bank voles. I observed that sex, time of the day and the excretion route of the metabolites affect the fecal steroid metabolite levels. These factors should always be taken into account for a meaningful and reliable interpretation of the results.

In this thesis I have provided new information on the overwintering strategies in boreal small mammals, by using the bank vole as a model species. Here, I have speculated as well as experimentally assessed some of the possible consequences of changing winter conditions for survival, behavior and the

initiation of breeding in winter-active boreal small mammals. Due to the immense complexity of ecosystem processes, drawing definitive conclusions of the impact of climate change on the success of species in adjusting their life histories to changing conditions is not possible. However, I suggest that the phenotypic plasticity and the opportunistic behavior of the small, short-lived species, like the bank vole, will mitigate some of the harmful effects caused by the adverse changes in their environmental conditions.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Pohjoisen pikkunisäkkään talvehtiminen muuttuvassa ilmastossa

Pohjoisilla alueilla talvi on vuodenajoista haastavin. Alueiden eläimet ovat kuitenkin sopeutuneet tähän kylmään ja pimeään ajanjaksoon monin eri tavoin. Syksyllä päivät lyhenevät ja vähenevä valonmäärä käynnistää eläimissä fysiologisia muutoksia, jotka vaikuttavat myös käyttäytymiseen. Yleisimpiä sopeumia talvioloihin ovat lisääntynyt rasvakerros, paksu ja hyvin lämpöä eristävä talviturkki sekä muutokset aineenvaihdunnassa. Eläinten talviaikainen käyttäytyminen poikkeaa usein muista vuodenajoista huomattavasti. Esimerkiksi monet pikkunisäkkäät ovat lisääntymisaikana aggressiivisia ja territoriaalisia, mutta talven tullen ne muuttavat käyttäytymistään suvaitsevammaksi ja sosiaalisemmaksi, mikä mahdollistaa yhteispesinnän. Yhteispesinnän oletetaan lisäävän pienten nisäkkäiden selviytymistä talven kylmyydestä.

Fysiologisten ja käyttäytymiseen liittyvien muutosten ja sopeumien lisäksi riittävä lumipeite on erityisen tärkeä monien lajien talvehtimismenestyksen kannalta. Erityisesti pienet nisäkkäät, kuten jyrsijät, ovat riippuvaisia lumipeitteen tuomasta suojasta kylmyyttä ja myös petoja vastaan. Lämpötila hangen alla on tasainen ja pysyttelee lähellä 0^0C huolimatta ilman lämpötilan voimakkaistakin vaihteluista. Maan ja lumipeitteen väliin muodostuva tila mahdollistaa pienten eläinten liikkumisen ja ravinnonhankinnan hangen suojissa.

Viimeaikaiset nopeat muutokset maapallon ilmasto-oloissa ovat kuitenkin herättäneet huolta pohjoisen luonnon kohtalosta tulevaisuudessa. Ilmastonmuutoksen on ennustettu vaikuttavan erityisen voimakkaasti maapallon pohjoisiin osiin, sillä lämpötila nousee pohjoisessa jopa kaksi kertaa nopeammin kuin muualla maailmassa. Tämänhetkisen ennusteen mukaan pohjoisilla leveysasteilla etenkin talvi tulee muuttumaan merkittävästi, ja talvista arvioidaankin tulevan lyhyempiä ja epävakaampia. Talviaikaisen sademäärän on ennustettu lisääntyvän mutta vähenevien pakkaspäivien seurauksena sateet tulevat yhä useammin vetenä eivätkä lumena. Vaihtelevat lämpötilat sekä veden ja lumen vuorottelu aiheuttavat maanpinnan jäätymistä, joka hankaloittaa monien eläinten liikkumista ja ravinnonhankintaa. Ilman pysyvää lumipeitettä pienet nisäkkäät altistuvat myös kylmyydelle sekä voimakkaalle sääolojen vaihteluille. Tämän lisäksi riski joutua petojen saaliiksi kasvaa, sillä pelkkä lakastunut kasvillisuus ei tarjoa tarpeeksi suojaa. Altistuminen monelle stressitekijälle samanaikaisesti voi huomattavasti heikentää pienten nisäkkäiden talvehtimismenestystä tulevaisuudessa. Muuttuvat elinolot voivat myös vaikuttaa pienten nisäkkäiden lisääntymiskauden alkamiseen talven jälkeen. Kevään nopean aikaistumisen on jo nyt huomattu aiheuttavan eriaikaisuutta eri eliölajien välillä. On myös mahdollista, että saman lajin eri sukupuolet reagoivat muuttuviin oloihin toisistaan poikkeavasti, mikä voisi aiheuttaa lisääntymismenestyksen huomattavaa heikentymistä. Monet fysiologiset ominaisuudet, kuten neurohormonaa-

linen säätely ovat keskeisessä osassa eläinten sopeutumisessa nopeasti muuttuviin ilmasto-oloihin.

Väitöskirjassani olen tutkinut Suomen yleisimmän nisäkkään, metsämyyrän (*Myodes glareolus*), talvehtimiskäyttäytymistä ja siihen vaikuttavia tekijöitä. Tämän lisäksi olen kokeellisesti pyrkinyt selvittämään ilmastonmuutoksen aiheuttamien talviolosuhdemuutosten mahdollisia vaikutuksia metsämyyrän fysiologiaan ja käyttäytymiseen. Selvitin myös eri ympäristötekijöiden, kuten lämpötilan ja ruoan laadun vaikutusta metsämyyrän lisääntymisen aloitukseen keväällä. Kaikki kokeeni sisälsivät hormonaalisia tutkimuksia, joita silmällä pitäen suoritin kokeen luotettavan analysointimenetelmän löytämiseksi hormonitasojen määritykseen metsämyyrän ulosteista.

Koeympäristönä tutkimuksissani toimivat Konneveden tutkimusaseman laboratoriot ja lämpösäädettävät ilmastokammiot, sekä suuret myyränpitävät ulkoaitaukset (0,25 ha per aitaus). Myyrien selviytymistä ja liikkeitä aitauksissa seurattiin elävänä pyytävillä loukuilla.

Tutkimukseni tulokset osoittivat, että talvehtivan myyräpopulaation sukupuolijakauma vaikuttaa metsämyyrien talvehtimisstrategioihin. Populaatioissa, joissa naaraita oli enemmän kuin koiraita, talvehtimisstrategia oli sosiaalinen, kun taas koirasvoittoisissa populaatioissa eläimet talvehtivat todennäköisemmin yksin. Lyhyen tutkimusjakson aikana eri strategioilla ei havaittu olevan merkitsevää vaikutusta selviytymiseen. Myöskään sukupuolien välillä ei selviytymisessä ollut eroja. Ilmastokammiossa suoritetussa kokeessa havaitsimme, että altistuminen voimakkaalle vuorokausittaiselle lämpötilan vaihtelulle aiheuutti muutoksia myyrien aktiivisuudessa sekä käyttäytymisessä petoriskitilanteissa. Altistuminen kahdelle stressitekijälle samanaikaisesti ei kuitenkaan aiheuttanut merkittävää nousua stressihormonitasoissa. Kevättutkimuksessani osoitin, että eri ympäristötekijät vaikuttavat naaraiden ja koiraiden lisääntymisen aloitukseen eri tavoin. Muuttuvissa ilmasto-oloissa tämä voi johtaa metsämyyrien heikkenevään lisääntymismenestykseen. Menetelmän validointitutkimuksessani osoitin, että sukupuoli, vuorokauden aika, sekä kuona-aineiden eritysreitti vaikuttavat kortikosteroni- (stressi) ja testosteronimetaboliittien pitoisuuksiin myyrän ulosteissa. Nämä muuttujat on tärkeä ottaa huomioon käytettäessä ulosteita hormonimääritystutkimuksiin metsämyyrällä.

Väitöskirjatutkimukseni sisältää uutta tärkeää tietoa metsämyyrän talvehtimiskäyttäytymisestä sekä antaa viitteitä muuttuvan ilmaston aiheuttamista mahdollisista seurauksista lumipeitteestä merkittävästi riippuvaisten pohjoisten nisäkäslajien talvehtimiselle sekä lisääntymisen aloitukselle.

REFERENCES

Aars J. & Ims R.A. 2002. Intrinsic and climatic determinants of population demography: the winter dynamics of tundra voles. *Ecology* 83: 3449-3456.

Ball G.F. & Ketterson E.D. 2008. Sex differences in the response to environmental cues regulating seasonal reproduction in birds. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 363: 231-246.

Banks P.B., Norrdahl K. & Korpimaki E. 2000. Nonlinearity in the predation risk of prey mobility. *Proc. Biol. Sci.* 267: 1621-1625.

Beldomenico P.M., Telfer S., Gebert S., Lukomski L., Bennett M. & Begon M. 2008. Poor condition and infection: a vicious circle in natural populations. *Proc. Biol. Sci.* 275: 1753-1759.

Berteaux D., Bergeron J-M., Thomas D.W. & Lapierre H. 1996. Solitude versus gregariousness: do physical benefits drive the choice in overwintering meadow voles?. *Oikos* 76: 330-336.

Berteaux D., Denis Réale, McAdam A.G. & Boutin S. 2004. Keeping Pace with Fast Climate Change: Can Arctic Life Count on Evolution? *Integr. Comp. Biol.* 44: 140-151.

Blumstein D.T. 2006. Developing an evolutionary ecology of fear: how life history and natural history traits affect disturbance tolerance in birds. *Anim. Behav.* 71: 389-399.

Boonstra R. 2004. Coping with changing northern environments: the role of the stress axis in birds and mammals. *Integr. Comp. Biol.* 44: 95-108.

Boonstra R. 2013. The ecology of stress: a marriage of disciplines. *Funct. Ecol.* 27: 7-10.

Boonstra R., Lane J.E., Boutin S., Bradley A., Desantis L., Newman A.E. & Soma K.K. 2008. Plasma DHEA levels in wild, territorial red squirrels: seasonal variation and effect of ACTH. *Gen. Comp. Endocrinol.* 158: 61-67.

Boonstra R., Dantzer B., Delehanty B., Fletcher Q.E. & Sheriff M.J. 2014. Equipped for Life in the Boreal Forest: The Role of the Stress Axis in Mammals. *Arctic* 67: 82-97.

Both C. & Visser M.E. 2001. Adjustment to climate change is constrained by arrival date in a long-distance migrant bird. *Nature* 411: 296-8.

Bronson F.H. 1989. *Mammalian reproductive biology.* University of Chicago Press.

Bronson F.H. 2009. Climate change and seasonal reproduction in mammals. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 364: 3331-3340.

Bujalska G. 1973. The role of spacing behaviour among females in the regulation of reproduction in the bank vole. *Journal Reprod. Fert.* 19: 465-474.

Butet A. & Delettre Y. 2011. Diet differentiation between European arvicoline and murine rodents. *Acta Theriologica* 56: 297-304.

Campbell N. & Reece J. 2005. *Biology. 7th.* Benjamin Cummings, San Francisco.

Cooper W.E., López P., Martín J. & Pérez-Mellado V. 2012. Latency to flee from an immobile predator: effects of predation risk and cost of immobility for the prey. *Behav. Ecol.* 23: 790-797.

Dantzer B., Boutin S., Humphries M.M. & McAdam A.G. 2012. Behavioral responses of territorial red squirrels to natural and experimental variation in population density. *Behav. Ecol. Sociobiol.* 66: 865-878.

Edut S. & Eilam D. 2004. Protean behavior under barn-owl attack: voles alternate between freezing and fleeing and spiny mice flee in alternating patterns. *Behav. Brain Res.* 155: 207-216.

Eilam D., Dayan T., Ben-Eliyahu S., Schulman I., Shefer G. & Hendrie C.A. 1999. Differential behavioural and hormonal responses of voles and spiny mice to owl calls. *Anim. Behav.* 58: 1085-1093.

Futuyma D.J. 2005. *Evolution.* Sinauer Associates, Sunderland, Mass.

Gartner K., Buttner D., Dohler K., Friedel R., Lindena J. & Trautschold I. 1980. Stress response of rats to handling and experimental procedures. *Lab. Anim.* 14: 267-274.

Gustafsson T., Andersson B. & Westlin L. 1980. Reproduction in a laboratory colony of bank vole, Clethrionomys glareolus. *Can. J. Zool.* 58: 1016-1021.

Gwinner E. 1986. *Circannual rhythms: endogenous annual clocks in the organization of seasonal processes.* Springer-Verlag Berlin.

Haemisch A., Guerra G. & Furkert J. 1999. Adaptation of corticosterone-but not beta-endorphin-secretion to repeated blood sampling in rats. *Lab. Anim.* 33: 185-191.

Hahn T.P., Boswell T., Wingfield J.C. & Ball G.F. 1997. Temporal flexibility in avian reproduction. In: Anonymous Current ornithology, Springer, pp. 39-80.

Hyvärinen H. 1984. Wintering strategy of voles and shrews in Finland. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 139-148.

Jylhä K., Fronzek S., Tuomenvirta H., Carter T.R. & Ruosteenoja K. 2008. Changes in frost, snow and Baltic sea ice by the end of the twenty-first century based on climate model projections for Europe. *Clim. Change* 86: 441-462.

Jylhä K., Ruosteenoja K., Räisänen J., Venäläinen A., Tuomenvirta H., Ruokolainen L., Saku S. & Seitola T. 2009. Arvioita Suomen muuttuvasta ilmastosta sopeutumistutkimuksia varten. *Ilmatieteenlaitos, raportteja 4* : 1-102.

Kaikusalo A. 1972. Population turnover and wintering of the bank vole, Clethrionomys glareolus (Schreb.), in southern and central Finland. *Ann. Zool. Fennici.* 9: 219-224.

Karasov W.H. & del Rio C.M. 2007. *Physiological ecology: how animals process energy, nutrients, and toxins.* Princeton University Press Princeton.

Klemme I., Eccard J.A. & Ylönen H. 2006. Do female bank voles (Clethrionomys glareolus) mate multiply to improve on previous mates? *Behav. Ecol. Sociobiol* 60: 415-421.

Koivula M., Koskela E., Mappes T. & Oksanen T. 2003. Cost of reproduction in the wild: Manipulation of reproductive effort in the bank vole. *Ecology* 84: 398-405.

Lovejoy T.E. & Hannah L.J. 2005. *Climate change and biodiversity / edited by Thomas E. Lovejoy & Lee Hannah.* Yale University Press.

Ludwig G.X., Alatalo R.V., Helle P., Linden H., Lindstroem J. & Siitari H. 2006. Short- and long-term population dynamical consequences of asymmetric climate change in black grouse. *Proc. R. Soc. Lond. , Ser. B: Biol Sci* 273: 2009-2016.

MacDonald D. 2001. *The Encyclopedia of mammals.* Andromeda Oxford Limited, United Kingdom, Oxford.

Marchand P.J. 1996. *Life in the cold: an introduction to winter ecology.* University Press of New England.

McEwen B.S. 2004. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Ann. N. Y. Acad. Sci.* 1032: 1-7.

McEwen B.S. & Wingfield J.C. 2003. The concept of allostasis in biology and biomedicine. *Horm. Behav.* 43: 2-15.

McNamara J. & Buchanan K. 2005. Stress, resource allocation, and mortality. *Behav. Ecol.* 16: 1008-1017.

Merritt J.F. & Zegers D.A. 2002. Maximizing survivorship in cold: thermogenic profiles of non-hibernating mammals. *Acta Theriol.* 47: 221-234.

Millar J.S. 2007. Nest mortality in small mammals. *Ecoscience* 14: 286-291.

Millar J.S. & Gyug L.W. 1981. Initiation of Breeding by Northern Peromyscus in Relation to Temperature. *Can. J. Zool.* 59: 1094-1098.

Mills S.C., Grapputo A., Jokinen I., Koskela E., Mappes T., Oksanen T.A. & Poikonen T. 2009. Testosterone-Mediated Effects on Fitness-Related Phenotypic Traits and Fitness. *Am. Nat.* 173: 475-487.

Moritz R.E., Bitz C.M. & Steig E.J. 2002. Dynamics of recent climate change in the Arctic. *Science* 297: 1497-1502.

Palme R. & Möstl E. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Zeitschrift fuer Saeugetierkunde (Germany).*

Parmesan C. 2006. Ecological and Evolutionary Responses to Recent Climate Change. *Annual Review of Ecology, Evolution and Systematics* 37: 637-669.

Putkonen J. & Roe G. 2003. Rain-on-snow events impact soil temperatures and affect ungulate survival. *Geophys. Res. Lett.* 30.

Rasmus S., Räisänen J. & Lehning M. 2004. Estimating snow conditions in Finland in the late 21st century using the SNOWPACK model with regional climate scenario data as input. *Annals of Glaciology* 38: 238-244.

Serreze M., Walsh J., Chapin Iii F., Osterkamp T., Dyurgerov M., Romanovsky V., Oechel W., Morison J., Zhang T. & Barry R. 2000. Observational evidence of recent change in the northern high-latitude environment. *Clim. Change* 46: 159-207.

Sharpe S. & Millar J.S. 1991. Influence on the variation in initiation of breeding in Peromyscus maniculatus. *Canadian Journal of Zoology/Revue Canadienne de Zoologie* 69: 698-705.

Sheriff M.J., Richter M.M., Buck C.L. & Barnes B.M. 2013. Changing seasonality and phenological responses of free-living male arctic ground squirrels: the importance of sex. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368: 20120480.

Smith S.M. & Vale W.W. 2006. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin. Neurosci.* 8: 383-395.

Spencer A.W. 1984. Food habits, grazing activities, and reproductive development of long-tailed voles, Microtus longicaudus(Merriam) in relation to snow cover in the mountains of Colorado. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 67-90.

Stankowich T. & Blumstein D.T. 2005. Fear in animals: a meta-analysis and review of risk assessment. *Proc. Biol. Sci.* 272: 2627-2634.

Stenseth N.C. & Mysterud A. 2002. Climate, changing phenology, and other life history traits: Nonlinearity and match-mismatch to the environment. *Proc. Natl. Acad. Sci. U S A* 99: 13379-13381.

Stien A., Ims R.A., Albon S.D., Fuglei E., Irvine R.J., Ropstad E., Halvorsen O., Langvatn R., Loe L.E., Veiberg V. & Yoccoz N.G. 2012. Congruent responses to weather variability in high arctic herbivores. *Biology letters* 8: 1002-1005.

Stocker T.F., Qin D., Plattner G., Tignor M., Allen S.K., Boschung J., Nauels A., Xia Y., Bex V. & Midgley P.M. 2013. Climate change 2013: The physical science basis. *Intergovernmental Panel on Climate Change, Working Group I Contribution to the IPCC Fifth Assessment Report (AR5)(Cambridge Univ Press, New York)* .

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

Trivers R.L. 1972. Parental investment and sexual selection. In: B. Campbell (ed.) Sexual selection and the descent of man 1871-1971: 136-179. Heinemann, London.

Viitala J. 1984. Stability of overwintering populations of *Clethrionomys* and *Microtus* at Kilpisjärvi, Finnish Lapland. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 109-112.

Visser M.E., Holleman L.J. & Caro S.P. 2009. Temperature has a causal effect on avian timing of reproduction. *Proc. R .Soc. Lond. , Ser. B: Biol. Sci.* 276: 2323-2331.

Visser M., van Noordwijk A., Tinbergen J. & Lessells C.M. 1998. Warmer springs lead to mistimed reproduction in great tits (Parus major). *Proc. R. Soc. Lond. , Ser B: Biol. Sci.* 265: 1867-1870.

Von Blanckenhagen F., Eccard J.A. & Ylönen H. 2007. Animal protein as a reproductive constraint in spring reproduction of the bank vole. *Ecoscience* 14: 323-329.

Webster A.B. & Brooks R.J. 1981. Social behavior of Microtus pennsylvanicus in relation to seasonal changes in demography. *J. Mammal. 62* : 738-751.

West S.D. & Dublin H.T. 1984. Behavioral strategies of small mammals under winter conditions: Solitary or social?. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 293-299.

Wingfield J.C. 2013. Ecological processes and the ecology of stress: the impacts of abiotic environmental factors. *Funct. Ecol.* 27: 37-44.

Wingfield J.C. & Hunt K.E. 2002. Arctic spring: hormone–behavior interactions in a severe environment. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* 132: 275-286.

Ydenberg R.C. & Dill L.M. 1986. The economics of fleeing from predators. *Adv. Study. Behav.* 16: 229-249.

Ylönen H. & Viitala J. 1985. Social organization of an enclosed winter population of the bank vole Clethrionomys glareolus. *Ann. Zool. Fennici* 22: 353-358.

Ylönen H. & Viitala J. 1991. Social overwintering and food distribution in the bank vole Clethrionomys glareolus. *Ecography* 14: 131-137.

Ylönen H., Sundell J., Tiilikainen R., Eccard J.A. & Horne T. 2003. Weasels'(Mustela nivalis nivalis) preference for olfactory cues of the vole (Clethrionomys glareolus). *Ecology* 84: 1447-1452.

ORIGINAL PAPERS

I

POPULATION SEX-RATIO AFFECTING BEHAVIOR AND PHYSIOLOGY OF OVERWINTERING BANK VOLES (*MYODES GLAREOLUS*)

by

Saana Sipari, Marko Haapakoski, Ines Klemme, Rupert Palme, Janne Sundell & Hannu Ylönen 2014

Manuscript

POPULATION SEX-RATIO AFFECTING BEHAVIOR AND PHYSIOLOGY OF OVERWINTERING BANK VOLES (*MYODES GLAREOLUS*)

Saana Sipari[1], Marko Haapakoski[1], Ines Klemme[1], Rupert Palme[3], Janne Sundell[2] and Hannu Ylönen[1]

1 Department of Biological and Environmental Science, Konnevesi Research Station, University of Jyväskylä, P.O.Box 35, FI-40014 Jyväskylä, Finland; 2 Lammi Biological Station, University of Helsinki, Pääjärventie 320, 16900 Lammi, Finland; 3 Institute of Physiology, Pathophysiology and Biophysics, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

ABSTRACT

Winter in the Boreal and polar regions is often the most challenging season for winter-active homeothermic species and requires physiological and behavioral adaptations. For example, many boreal rodents, like voles, are territorial during the breeding season but during winter they become more social and aggregate for more energy efficient thermoregulation. Communal winter nesting and social interactions are considered to play an important role for the winter survival of these species, yet the topic is relatively little explored. Based on earlier studies, females are suggested to be the initiators of winter aggregations and they are also reported to survive better than males. This could be partly due to the higher social tolerance observed in overwintering females, than males. Hormonal status could also affect the winter behavior and survival. For instance, chronic stress can have a negative effect on survival whereas high gonadal hormone levels, such as testosterone, often induce aggressive behavior. To test the hypothesis that the winter survival of females in boreal rodents is better than that of males, and assess the role of females in creating and maintaining the winter aggregations, we generated bank vole (*Myodes glareolus*) populations of three different sex ratios (male-biased, female-biased and even, constant density) under semi-natural conditions. We monitored survival, spatial behavior and hormonal status (stress and testosterone levels) during two winter months. We observed no significant differences in survival between the sexes or among populations with differing sex ratios. The degree of movement area overlap was used as an indicator of social tolerance and potential communal nesting. Individuals in male biased population type showed tendency to be solitary whereas in female biased populations there was an indication of winter aggregation. Females living in male biased populations had significantly higher stress levels than the females from the other populations. The female biased sex-ratio induced winter breeding and elevated testosterone levels in males. Thus, our results suggest that the sex-ratio of the overwintering population can lead to divergent overwintering strategies in bank voles.

Key words: Overwintering, survival, winter aggregation, communal nesting, social interactions, population sex-ratio, hormonal status

INTRODUCTION

Overwintering strategies of different organisms in boreal and arctic regions can be divided roughly in three categories; migration, hibernation and resistance. Migration is most common among birds, whereas reptiles and amphibians, as well as some mammals rely on hibernation. However, surprisingly many animals remain active throughout the winter, despite the challenging environmental conditions. For instance, northern cervids, like moose, deer and caribou are very well equipped against cold with their large body size, thick winter pelage and fat reserves. But not all winter-active animals are large and armed with similar traits. In fact, many homeothermal winter-active animals are small mammals like rodents and shrews. Unlike large animals, their winter survival is directly and strongly dependent on snow cover. Under the snow pack these small animals exploit the subnivean space formed between the ground, withered vegetation and the snow for moving and foraging, but most importantly for protection against cold and predators. However, besides the importance of the physical protection provided by snow cover, also physiological and behavioral overwintering adaptations are required. Particularly, the hypothalamic-pituitary-adrenal axis (HPA axis) is essential in equipping mammals to endure the harsh conditions of northern winter (Boonstra 2004, Boonstra *et al.* 2014). It regulates several substantial body processes such as energy metabolism, reproduction, growth, immune system and stress reactions (Boonstra 2004, Campbell and Reece 2005). The function of the endocrine system during winter varies amongst species but in small winter-active rodents decreasing day length and temperature are known to induce changes e.g. in fur thickness and metabolism. Also, many small mammals reduce their body mass to lower energy needs, and often all reproductive functions cease in order to allocate energy for survival (Hyvärinen 1984, Feist 1984, Hansson 1990, Marchand 1996, Merritt and Zegers 2002, Aars and Ims 2002). In addition to these physiological changes, the HPA axis is considered to be responsible of changes in winter time behavior as well (Boonstra *et al.* 2014). Some species that are solitary and territorial during the breeding season are known to become more social during winter, and to construct communal nests (Webster and Brooks 1981, West and Dublin 1984, Ylönen and Viitala 1985, Ylönen and Viitala 1991, Merritt and Zegers 2002). This is often related to the seasonal decrease in gonadal steroid levels, e.g. testosterone in males (Bronson 1985, Boonstra *et al.* 2014).

The increased social tolerance and the social interactions in the overwintering population are considered to play an important role in the winter survival of many small rodents. The main benefit gained from aggregation seems to be the social thermoregulation, as cold is one of the main stressors during winter. Huddling keeps the nest temperature higher which is more energy efficient compared to individual nesting (Sealander 1952, Vickery and Millar 1984, but see Berteaux *et al.* 1996). It has been observed in Taiga voles (*Microtus xanthognathus*) that in order to prevent nest temperatures from dropping, foraging bouts are timed between individuals in a way that the nest

is never left empty (Wolff and Lidicker Jr 1981) However, in case of scarce food resources the communal nesting creates an inevitable trade-off between energy gain and energy consumption (West and Dublin 1984). Further, the degree of communal nesting seems to vary between species, years, habitats and winter characteristics (Viitala 1984, West and Dublin 1984) comprising incomplete understanding of the actual determinants of successful overwintering. Due to the nature of their overwintering environment, i.e. under the snow pack, the role of social interactions and behavior on winter survival in small winter-active ground-dwelling mammals is relatively little explored.

In order to define the impact and importance of behavior and social dynamics on overwintering survival in a rodent species with highly territorial behavior during the breeding season, we performed a field enclosure experiment at the onset of winter. As a model species we used the bank vole (*Myodes glareolus*), one of the most common winter-active boreal rodents. Bank voles are short lived, granivorous-omnivorous small rodents. During the breeding season, from April-May to September, they are territorial. Female bank voles, like all *Myodes* females, defend their exclusive breeding territories, whereas the home ranges of males are larger and overlap with each other and with several females' territories (Bujalska 1973). However, it appears that during winter their social tolerance increases and similar to many *Microtus* species they exploit communal winter nests (Ylönen and Viitala 1985, Ylönen and Viitala 1991). Interestingly, females are reported to survive better over winter than males (Ylönen and Viitala 1985, Klemme *et al.* 2008). Their smaller body size, and thus, lower absolute energetic need is assumed to be a major factor behind this. In addition, it has been suggested by Ylönen et al. (1995) that females with their better "social skills" form the core of winter aggregations and that this could play an important role in the overwintering success of the whole overwintering population. Based on the experiment on odor preference, also Ferkin & Seamon (1987) suggested that overwintering groups of meadow voles (*Microtus pennsyvanicus*) might be female biased, as non-breeding meadow vole females showed preference for the odor of female conspecifics, whereas males showed no preference and displayed more antagonistic acts against other males, than females against female conspecifics. Thus, male-male aggressive interactions may preclude males from joining the same winter aggregation. A tendency for aggressive behavior could be reflected in higher hormonal levels related to competitive ability and aggression, like testosterone, as well as high stress levels if there are many competing males present. Being isolated from communal nests would also mean higher energetic costs in terms of thermoregulation and thus, possibly increased stress levels caused by the cold. This could decrease the survival possibility of solitary overwintering individuals. However, maintaining testosterone levels high enough for breeding during winter could be beneficial, because winter breeding occurs occasionally in many small boreal rodent species (Hansson 1984; Jannett 1984; Kaikusalo & Tast 1984). As the winter mortality of small rodents is relatively high, trading off survival for reproduction may increase fitness.

To test the hypothesis of better winter survival of females and assess their role as initiators of winter aggregations, as well as the impact of population structure and hormonal status on winter time behavior and survival, we generated three different population types in semi-natural outdoor enclosures in late October, before the onset of winter: Female biased population type (F), male biased population type (M) and even sex ratio population type (E). Spatial and temporal variation in the sex ratio is relatively common in natural animal populations and can be caused by several factors depending on the species (Werken and Charnov 1978). To estimate social dynamics, we monitored space use of the voles and the degree of movement area overlap as an indicator of social tolerance and decreased level of aggression between individuals, which could imply communal nesting. We also observed how the hormonal status of individuals affects their behavior and survival, and further, whether the population type (F, M or E) affects the hormonal status. For this we monitored stress and testosterone levels in the form of fecal steroid metabolites. Stress was monitored in both sexes, but testosterone only in males.

We hypothesized that voles in female biased populations would survive best and express the highest social tolerance and the lowest stress levels. Male biased populations were expected to survive worst and have the highest stress levels due to high encounter probability and low tolerance between males. Also, if females are the ones maintaining the communal winter nests it is likely that the total number of communal nests or aggregations would be lower in male biased populations due to the shortage of females. Bank voles are suggested to aggregate in groups of 2-5 individuals (Ylönen and Viitala 1991), so this could mean that some of the males will be left out from the aggregations. If these surplus males were yet attempting to join these winter nests, it could disturb the nesting and increase stress level also in females. Males with high levels of testosterone were hypothesized to express low survival. High testosterone would likely maintain the social intolerance and aggression and probably lead to isolation from communal nests. This would expose the individual to cold stress. Additionally, testosterone is known to be immunosuppressive, and maintaining high testosterone level is energy consuming (Mills *et al.* 2009, Boonstra *et al.* 2014).

MATERIAL AND METHODS

Experimental animals

All animals used in this study (72 males and 72 females) were born in the laboratory during April-July 2012 at Konnevesi research station. One month before the actual experiment commenced, all voles were transferred to a greenhouse for acclimatizing to outdoor temperatures and light rhythms. The greenhouse was not heated and the temperature in the greenhouse was similar to outdoors. Voles were housed separately in standard mouse cages (43×26×15

cm) with wood shavings and hay as bedding. Standard mice pellets (Labfor R36, Lantmännen) and water were provided *ad libitum*.

Experimental design

The experiment was carried out in 12 large outdoor enclosures (0.25ha each) from late October to mid December 2012. Voles were divided into three population types; female biased populations (F), male biased populations (M) and even sex-ratio populations (E). Each population type was replicated in four enclosures with 12 animals in each. In sex biased populations the ratio of the sexes was 8: 4, and in even population half and half (6: 6). Populations were distributed randomly among the 12 enclosures. Before releasing the voles into enclosures all individuals were weighed. To identify the animals we used ear tags with individual numbering. We also collected fecal samples for the evaluation of corticosterone metabolite levels. In males, testosterone metabolites were also measured (see *Fecal sampling and analysis*). At the beginning of the experiment there were no differences in corticosterone metabolite levels between groups (males: $F_{2,48} = 0.325$, p = 0.724; females: $F_{2,46} = 1.455$, p = 0.244. Females have naturally significantly lower basal level, ergo, separate analysis), nor in testosterone metabolite levels ($F_{2,53} = 0.356$, p = 0.703).

During the experiment voles were monitored by multiple live trappings. In each enclosure we had 25 multiple-capture live traps (Ugglan special®, Grahnab AB, Hillerstorp, Sweden) distributed evenly in 5x5 grids. In total we performed three trapping sessions; two for monitoring the survival and space use in different populations, and one for fecal sample collection. Sunflower seeds were used as bait. The first session was carried out during early November, one week after releasing the animals into the enclosures, in order to monitor the survival and space use. Trapping was conducted three times per day (early morning, afternoon, and late evening) with ten trap checks in total. The second trapping was conducted for fecal sample collection in late November (see *Fecal sampling and analysis*). The third and last trapping session was performed in mid-December again to monitor the space use and survival, similar to the first session. This trapping session followed the same protocol as the first one, but this time we also weighed the captured individuals with Pesola spring scales. Permanent snow came in the beginning of December, and during the last trapping session the average snow depth was 25-30 cm.

Fecal sampling and analyses

To collect the samples in laboratory conditions, before the animals were released to the enclosures, each vole was temporarily moved to an empty (no beddings) mouse cage (43×26×15 cm). After the vole defecated, it was returned to its home cage. This usually happened fast, and took a maximum of one hour. After this, feces were collected in empty Eppendorf-tubes (1.5 ml, one tube per individual) from the sampling cage using tweezers. Samples were stored at -20

°C. Fecal samples were collected from voles released into 9 enclosures out of 12 in total (54 males and 54 females).

One month after the release, we performed a trapping session for fecal sample collection. All traps used for this session were new and unused. Samples were collected from the floor of the traps with tweezers to plastic Eppendorf tubes and the trap was wiped clean with paper towels after each sampling. The feces were usually very dry and did not stain the trap. Also, fecal samples are not highly sensitive to contamination. With larger animals, samples are often collected directly from the ground so this method is well suited for field conditions as ultimate sterility is not required. However, samples soaked in urine were not used as it can distort the results (Sipari, Palme, Ylönen, unpublished results). Samples were stored at -20 C. When two or more voles were inside the same trap simultaneously we did not collect the samples. This happened twice. The traps were checked every three hours, three times per day, and one day per enclosure. In total, samples were collected from nine enclosures, three enclosures for each population type (see above). This took four days. No overnight trapping was done in order to keep the time the voles spend captured in the traps as low as possible, to avoid possible stress caused by the captivity showing in the samples and affecting the results. Trapping data were not used for evaluating space use and survival.

All fecal samples were analyzed using 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA), with methods described in Touma et al. 2003. This specific EIA has been validated and proven suitable for measuring fecal corticosterone metabolites in bank voles (Sipari, Palme, Ylönen, unpublished results). In that validation experiment it was shown that males excrete on average 70 % of corticosterone metabolites via feces, whereas females excrete only around 50 %. Due to that significant difference we adjusted the measured corticosterone metabolite levels to the estimated value of 100% for both sexes for meaningful comparisons in our statistical analyses and in the illustrations for this paper. To measure fecal testosterone metabolites in males we used EIA for 17ß-hydroxyandrogen, first described by Palme & Möstl (1994), and validated for bank voles by Sipari, Palme and Ylönen (Unpub.). All laboratory analyses were performed in the University of Veterinary Medicine, in Vienna, Austria.

Statistical analyses

Statistical analyses were performed using R 3.0.3 and IBM SPSS Statistics 20. Survival was analysed using generalized linear mixed model (GLMM) with binomial distribution, and for analysing space use, corticosterone metabolite, testosterone metabolite and body mass we used linear mixed model (Gaussian distribution) fitted with restricted max. likelihood (REML).

To test if survival was affected by time (first and third trapping session), sex and population type (F, M, E), those were set as fixed factors, and individual nested within enclosure as random factor. We also tested the time

points separately but that did not change the final result. The space use data was first analysed using Ranges 6 program (Anatrack ltd. Wareham, UK), using 100% convex polygon for calculating movement areas. For statistical testing, time, sex and population type were used as fixed factors and individual nested within enclosure as random factor. For better fit the data was log-transformed. To test the effect of time on the degree of movement area overlap we set time as fixed factor and individual nested within enclosure as random factor. We also tested if there was a difference in the tendency to overlap female or male area between population types between the sexes. We tested sexes separately against different overlapping types (e.g. males overlapping females, males overlapping males etc.) We set time and population type as fixed factors and individual nested within enclosure as random factor. For testing corticosterone metabolite concentrations, sex, population type and time were set as fixed factors and individual nested within enclosure as random factor. Additionally, for a more meaningful comparison of the population types we analysed the sexes separately and used only data collected from the field, as that was our main interest. For that we used population type as fixed factor and individual nested within enclosure as random factor. The data was log-transformed. To test the possible effect of stress on survival we compared the corticosterone metabolite concentrations measured in the field (late November) between individuals who were still alive in December and individuals who had died by December. Survival status (dead or alive) was set as fixed factor and individual nested within enclosure as random factor. Sexes were tested separately. Testosterone metabolite levels were tested first with time and population type as fixed factors and individual nested within enclosure as random factor. Similar to above, for more meaningful comparison between population types we then used only the data collected from the field. For better fit the data was log-transformed. To test if there was a difference in testosterone metabolite levels measured in field (late November) between individuals alive and dead by December we used survival status as fixed factor and individual nested within enclosure as random factor. Body mass was tested with time sex and population type as fixed factors and individual and enclosure as random factors. To test if the body mass of the individuals at the beginning of the experiment would reflect survival probability we compared the starting body masses of individuals still alive in December and individuals who had died by December. Survival status and sex were set as fixed factors and individual nested within enclosure as random factor. For model selection we used Akaike information criterion (AIC), the model with the lowest AIC value was selected for the analyses.

RESULTS

Survival

Sex had no significant effect on survival ($F_{1,120}$ = 1.1942, p = 0.277), nor did the population type ($F_{2,8}$ = 1.470, p = 0.293). In general, the male biased populations

tended to survive worse than even sex-ratio or female biased populations (Fig. 1), but not significantly. Time, on the other hand, had a clear effect, as expected. One week after release the average survival was 66.7%, but it decreased significantly by December, when only 32.3% of the animals were still alive ($F_{1,131}$ = 49.1298 p < 0.001). No significant interactions were detected.

Space use in the enclosures

The movement areas inside the enclosures were significantly larger for males than females ($F_{1,72}$ = 8.982, p = 0.003). In November the average movement area in males was 156 ± 21 m^2 and in females 95.5 ± 21.8 m^2 (values reported with standard errors). By December the areas had decreased significantly in both sexes ($F_{1,33}$ = 9.772, p = 0.004 in males 77 ± 24.2 m^2, and in females 28 ± 9.6 m^2). There were no significant differences between populations ($F_{2,8}$ = 2.153, p = 0.179).

The degree of movement area overlap decreased significantly from November till December ($F_{1,57}$ = 19.094, p < 0.001). We also tested if there was a difference in the tendency to overlap female or male areas between population types and sexes. The population type significantly affected the ratio of males overlapping male areas ($F_{2,8}$ = 10.527, p = 0.006), as males from male biased population seemed to avoid overlapping with other males (Fig. 2). By December the overlapping percent in male biased populations was 0% (male-male overlap, pairwise comparison: E vs M; Z = 4.763, p < 0.001, F vs M; Z = 5.738, p < 0.001, E vs F; Z = -0.591, p = 0.992). However, there were no differences in the tendency of males to overlap female areas between population types ($F_{2,7}$ = 2.421, p = 0.159). The population type did not affect the overlapping tendency or direction in females (females overlapping males: $F_{2,7}$ = 2.100, p = 0.193, females overlapping females: $F_{2,8}$ = 1.724, p = 0.239).

Fecal steroid metabolites

Sex had a significant effect on stress levels measured as fecal corticosterone metabolite concentrations ($F_{1,24}$ = 43.029, p < 0.001). However, this was likely due to the naturally lower basal corticosterone level in females (Sipari, Palme, Ylönen, unpublished results) rather than a difference in the stress experienced. After spending 1 month in the enclosures the stress levels had increased significantly ($F_{1,24}$ = 6.183, p = 0.020). There were no significant interactions. For a more meaningful comparison between populations we tested the sexes separately and compared only the stress levels measured from the field. The population type affected significantly on stress levels in females ($F_{2,6}$ = 5.248, p = 0.048) but not in males $F_{2,5}$ = 1.067, p = 0.411, Fig. 3). In females, pairwise comparison showed that the M populations had significantly higher stress level than the F populations (Z = -3.186, p = 0.004) but the other comparisons were not significant. Stress levels during November did not affect the survival

observed in December (males: $F_{1,13}$ = 0.942, p = 0.349; females: $F_{1,7}$ = 2.812, p = 0.138).

By the end of November, testosterone metabolite levels had decreased significantly ($F_{1,18}$ = 10.119, p = 0.005), except for female biased populations, where the testosterone metabolite levels actually increased, though not significantly (Fig. 4). The population type had a significant effect on testosterone metabolite levels ($F_{2,19}$ = 9.099, p = 0.002). In pairwise comparisons the levels were significantly higher in female biased than male biased or even sex-ratio populations (E vs. F; Z = -3.241, p = 0.003, F vs. M; Z = 4.199, p < 0.001). The E and the M population had no significant difference (Z = 0.806, p = 0.699). Testosterone metabolite levels measured in the field in November affected the survival observed in December ($F1,13$ = 6.000, p = 0.029). Individuals who were still alive by December had significantly lower testosterone metabolite levels than those who had died by then (Z=-2.449, p = 0.014).

Body mass

There were no significant differences in the body mass between different populations throughout the experiment ($F_{2,8}$ = 0.406, p = 0.679). Males were significantly heavier than females ($F_{1,117}$ = 4.803, p = 0.030). The body mass of the animals decreased significantly after the release ($F_{1,28}$ = 16.892, p < 0.001). Body mass in the beginning of the experiment had a significant effect on the survival observed in December ($F_{11,115}$ = 4.460, p = 0.037). In pairwise comparisons females that survived better were heavier at the beginning of the experiment than those that had died by December (Z = 3.017, p = 0.014). In males there was no significant difference (Z = -0.368, p = 0.983).

DISCUSSION

Our results suggest that the sex-ratio of the overwintering population may affect the behavior and overwintering strategies of bank voles. In our experiment we were not able to verify earlier findings of higher winter survival in females compared to males. However, our results suggest that females may play an important role in creating and maintaining the winter aggregations and communal winter nests, as the degree of overlapping movement areas indicating winter aggregation was higher in female biased and even sex-ratio population types than male biased populations. Also, the sex-ratio of the population affected significantly the hormonal status of the individuals. Voles living in male biased populations tended to have higher stress levels than individuals from other population types, whereas in female biased populations the males showed significantly higher testosterone levels compared to other populations. High testosterone levels affected negatively on survival, but the differences in stress levels did not result in different survival as expected.

Individuals in male biased populations tended to have a lower survivability than in other population types but the difference was not significant. However, the overlapping of the movement areas between individuals indicating social interactions and perhaps a tendency for communal nesting, was 0 % in male biased populations in December, suggesting divergent social dynamics compared to even sex-ratio and female biased populations.

The total sizes of the movement areas within a gender did not differ between population types, which indicates that avoiding overlapping movement areas with others in male biased populations was indeed intentional rather than derived by a chance. Also, stress levels measured as fecal corticosterone metabolite concentration were highest in male biased populations although in pairwise comparisons between population types only females showed significant differences. However, the individual variation in corticosterone metabolite levels is high in bank voles, especially in males, which makes it challenging to obtain statistically significant results (Sipari, Palme, Ylönen unpublished data). Nevertheless, the elevated stress levels and the lack of overlapping areas could indicate a solitary overwintering strategy and antagonistic social dynamics between individuals.

Aggression is often related to testosterone in males (Bronson 1989), and thus it was surprising to notice that the fecal testosterone metabolite concentrations were actually lowest in males from male biased populations. The difference was significant between female biased population type but not with the even sex-ratio population type. This suggests that the winter time aggressiveness cannot be solely testosterone induced. There are similar observations in some other rodents showing that in seasonal environments the short days reduce testosterone levels but induce aggressive behavior (Jasnow *et al.* 2000, Jasnow *et al.* 2002). In red squirrels (*Tamiasciurus hudsonicus*) aggressiveness during the non-breeding season has been suggested to be related to the high concentrations of dehydroepiandrosterone (DHEA), an androgen precursor (Boonstra *et al.* 2008). DHEA is also linked to aggression in non-breeding songbirds (Soma *et al.* 2008). Density and territorial conflicts appear to increase DHEA levels, and it is noticed to be higher in stressed animals (Boonstra *et al.* 2008). This could explain the assumed antagonistic behavior occurring even with low testosterone levels in males from the male biased populations in our experiment. Unlike testosterone the DHEA does not have similar negative effects on body condition, such as immunosuppression and increased energy metabolism (Soma *et al.* 2008, Boonstra *et al.* 2014).

Winter time aggression in red squirrels was explained by the attempt to defend their food stores as food is often highly limited during winter (Dantzer *et al.* 2012). This could also be the case with the voles in the male biased populations. Males are larger than females and thus their absolute energy needs are higher. In populations with high male density and restricted food resources, staying solitary may overcome the benefits of communal nesting (West & Dublin 1984). It has been reported that the food distribution inside a given area does not affect the overwintering strategy in bank voles (Ylönen and Viitala

1991), but perhaps total food availability could. The higher stress levels observed in male biased populations could be derived from territorial conflicts as well as cold stress due to the possible solitary nesting.

Female biased sex ratio did not increase the survival probability, at least not during early winter. However, compared to male biased populations the degree of males' movement area overlap with other males was significantly higher, suggesting more amicable social interactions. Also the tendency of females to overlap the areas of other females and males was higher but not significantly. Further, the higher female density in the population resulted in significantly higher fecal testosterone metabolite concentrations in males compared to males from other population types. During the acclimatization period in the green house, before the actual experiment and division into different populations, males had already lowered their testosterone production towards the overwintering levels. In the enclosures, by the end of November, males in male biased populations and even sex-ratio populations had decreased their testosterone levels even further, but males in female biased populations showed quite the opposite trend. On average, males in female biased populations had testosterone levels high enough for reproduction in late November. During the last trapping session in December, we observed one female visibly pregnant and several receptive females (vaginal opening used as an indication) in female biased populations.

The presumed social aggregation and possible communal nesting combined with winter breeding in female biased populations differs quite drastically from the overwintering strategy observed in male biased populations. The ultimate reason for this, however, is not clear. Perhaps the benefits of communal nesting combined with the lower food consumption of females provide circumstances favorable enough for restoring breeding condition and reproducing during winter (Ylönen and Viitala 1985). In general, high testosterone level in males had a negative effect on survival. However, the observed high testosterone levels of males in female biased populations did not result in lower survival on population level compared to other population types, at least not during the early winter. Also, there were no observable differences in female space use between populations, which would indicate territoriality in female biased populations due to the restored breeding activity. Neither the elevated testosterone levels seemed to cause observable (in terms of movement area overlaps) aggressive behavior.

Winter breeding is known to occur occasionally in many vole species (Jannett 1984, Kaikusalo and Tast 1984, Ylönen and Viitala 1985) but the factors enabling or causing this phenomenon are not always clear. High food abundance strongly correlates with the probability of winter breeding, but it does not explain all of the observed occasions (Jannett 1984). Population density is considered to be an important factor, as winter breeding is often reported to occur at low or increasing population densities (Jannett 1984). Our results, however, suggest that perhaps instead of population density *per se*, the female density and the low male-male competition in the population could play a key role inducing winter breeding.

In the populations with even sex-ratio the degree of overlapping movement areas was quite similar to female biased population, suggesting that social aggregation occurred also in even sex-ratio populations. However, the observed movement areas of females were so small by December that interpreting the results of females' movement area overlap should be done cautiously. The hormonal responses in even sex-ratio populations showed no prominent trends compared to the other population types. Perhaps in populations with a balanced female-male density the number of females is high enough to enable a certain degree of communal nesting, but not necessarily favorable for inducing winter breeding.

Body mass during winter has been suggested to play an important role in the survival of small rodents (Marchand 1996, Aars and Ims 2002). Lowering body mass is a commonly observed overwintering adaptation in small winter-active rodents, as it lowers the absolute energy requirement during the season with often limited food availability (Marchand 1996). However, the smaller body size comes with the disadvantage of an increased cooling rate due to the relatively larger surface area. In our experiment the body masses did not differ between different population types, but in general, females with lowest autumn body weight survived worse than the heavier individuals. In males it was the other way around but the difference was not significant. Thus, it appears that the trade-off between decreased energy requirement due to smaller body mass, and increased energy consumption via heat loss due to the increased surface-area-to-volume ratio needs to be adjusted carefully to minimize the costs.

Conclusion

It is intriguing how the gender distribution of an overwintering population can lead to such divergent overwintering strategies in bank voles, while living in almost identical habitats and experiencing the same environmental conditions. To determine whether one of the strategies is better than the other in terms of higher survival or direct fitness gain would require more extensive long term experiments. Based on the trends observed in our experiment it seems possible that the solitary overwintering strategy presumably exploited in male biased populations could eventually result in lowest survival, as hypothesized, though we could not verify this during this short term early winter experiment. On the other hand, in the case of a rapid decrease in food abundance the communal nesting could turn out to be the unfavorable strategy. Particularly, the energetically demanding winter breeding could easily result in high mortality in the population in case of suddenly deteriorated environmental conditions. Nevertheless, our results show evident behavioral plasticity, as well as opportunism in the overwintering strategies in bank voles under northern winter conditions.

REFERENCES

Aars J. & Ims R.A. 2002. Intrinsic and climatic determinants of population demography: the winter dynamics of tundra voles. *Ecology* 83: 3449-3456.

Berteaux D., Bergeron J-M., Thomas D.W. & Lapierre H. 1996. Solitude versus gregariousness: do physical benefits drive the choice in overwintering meadow voles?. *Oikos* 76: 330-336.

Boonstra R. 2004. Coping with changing northern environments: the role of the stress axis in birds and mammals. *Integr. Comp. Biol.* 44: 95-108.

Boonstra R., Lane J.E., Boutin S., Bradley A., Desantis L., Newman A.E. & Soma K.K. 2008. Plasma DHEA levels in wild, territorial red squirrels: seasonal variation and effect of ACTH. *Gen. Comp. Endocrinol.* 158: 61-67.

Boonstra R., Dantzer B., Delehanty B., Fletcher Q.E. & Sheriff M.J. 2014. Equipped for Life in the Boreal Forest: The Role of the Stress Axis in Mammals. *Arctic* 67: 82-97.

Bronson F.H. 1985. Mammalian reproduction: An ecological perspective. *Biol. Reprod.* 32: 1-26.

Bronson F.H. 1989. *Mammalian reproductive biology.* University of Chicago Press.

Bujalska G. 1973. The role of spacing behaviour among females in the regulation of reproduction in the bank vole. *Journal Reprod. Fert.* 19: 465-474.

Campbell N. & Reece J. 2005. *Biology. 7th.* Benjamin Cummings, San Francisco.

Dantzer B., Boutin S., Humphries M.M. & McAdam A.G. 2012. Behavioral responses of territorial red squirrels to natural and experimental variation in population density. *Behav. Ecol. Sociobiol.* 66: 865-878.

Feist D.D. 1984. Metabolic and thermogenic adjustments in winter acclimatization of subarctic Alaskan red-backed voles. In: Merritt J.F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 131-137.

Ferkin M.H. & Seamon J.O. 1987. Odor preference and social behavior in meadow voles, Microtus pennsylvanicus: seasonal differences. *Can. J. Zool.* 65: 2931-2937.

Hansson L. 1990. Ultimate factors in the winter weight depression of small mammals. *Mammalia* 54: 397-404.

Hyvärinen H. 1984. Wintering strategy of voles and shrews in Finland. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 139-148.

Jannett F.J.,Jr. 1984. Reproduction of the montane vole, Microtus montanus, in subnivean populations. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 215-224.

Jasnow A.M., Huhman K.L., Bartness T.J. & Demas G.E. 2000. Short-Day Increases in Aggression Are Inversely Related to Circulating Testosterone Concentrations in Male Siberian Hamsters (< i> Phodopus sungorus). *Horm. Behav.* 38: 102-110.

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Jasnow A.M., Huhman K.L., Bartness T.J. & Demas G.E. 2002. Short Days and Exogenous Melatonin Increase Aggression of Male Syrian Hamsters (< i> Mesocricetus auratus). *Horm. Behav.* 42: 13-20.

Kaikusalo A. & Tast J. 1984. Winter breeding of microtine rodents at Kilpisjärvi, Finnish Lapland. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 243-252.

Klemme I., Ylonen H. & Eccard J.A. 2008. Long-term fitness benefits of polyandry in a small mammal, the bank vole Clethrionomys glareolus. *Proc. Biol. Sci.* 275: 1095-1100.

Marchand P.J. 1996. *Life in the cold: an introduction to winter ecology.* University Press of New England.

Merritt J.F. & Zegers D.A. 2002. Maximizing survivorship in cold: thermogenic profiles of non-hibernating mammals. *Acta Theriol.* 47: 221-234.

Mills S.C., Grapputo A., Jokinen I., Koskela E., Mappes T., Oksanen T.A. & Poikonen T. 2009. Testosterone-Mediated Effects on Fitness-Related Phenotypic Traits and Fitness. *Am. Nat.* 173: 475-487.

Palme R. & Möstl E. 1994. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. *Advances of Steroid Analysis '93" (Ed. S. Görög), Akadémiai Kiadó, Budapest* : 111-117.

Sealander J.A.,Jr. 1952. The Relationship of Nest Protection and Huddling to Survival of Peromyscus at Low Temperature. *Ecology* 33: 63-71.

Soma K.K., Scotti M.L., Newman A.E., Charlier T.D. & Demas G.E. 2008. Novel mechanisms for neuroendocrine regulation of aggression. *Front. Neuroendocrinol.* 29: 476-489.

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

Vickery W.L. & Millar J.S. 1984. The energetics of huddling by endotherms. *Oikos* 43: 88-93.

Viitala J. 1984. Stability of overwintering populations of *Clethrionomys* and *Microtus* at Kilpisjärvi, Finnish Lapland. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 109-112.

Webster A.B. & Brooks R.J. 1981. Social behavior of Microtus pennsylvanicus in relation to seasonal changes in demography. *J. Mammal.* 62: 738-751.

Werken J.,H & Charnov E.,L. 1978. Facultative sex ratios and population dynamics. *Nature* 272: 349-350.

West S.D. & Dublin H.T. 1984. Behavioral strategies of small mammals under winter conditions: Solitary or social?. In: Merritt J., F. (ed.), *Winter Ecology of Small Mammals*, Carnegie Museum of Natural History,Spec. Pub 10, Pittsburgh, pp. 293-299.

Wolff J. & Lidicker Jr W.Z. 1981. Communal winter nesting and food sharing in taiga voles. *Behav. Ecol. Sociobiol.* 9: 237-240.

Ylönen H. & Viitala J. 1985. Social organization of an enclosed winter population of the bank vole Clethrionomys glareolus. *Ann. Zool. Fennici* 22: 353-358.

Ylönen H. & Viitala J. 1991. Social overwintering and food distribution in the bank vole Clethrionomys glareolus. *Ecography* 14: 131-137.
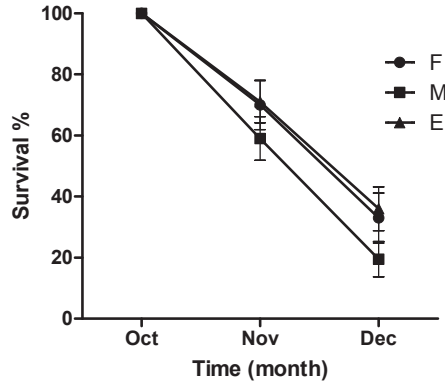
FIGURE 1   Survival rates of different population types during the experiment (LS-means ± SE). (F = female biased population type, M = male biased population type, E = even sex-ratio population type).
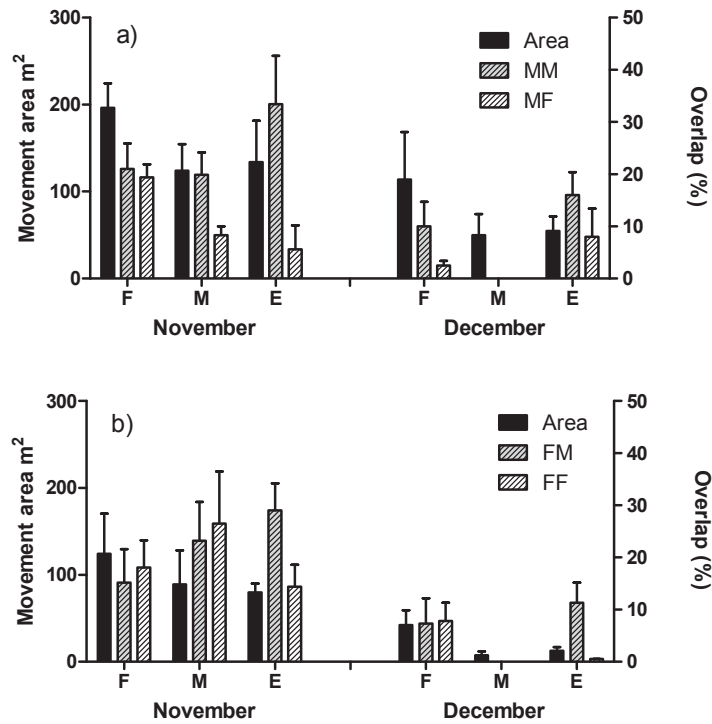
FIGURE 2   The sizes of the movement areas and the degree of overlapping areas in males (a) and in females (b) during November and December in different population types. MM = males overlapping males, MF = males overlapping females, FM = females overlapping males, FF = females overlapping females. The scale for "Area" is on left y-axis, and the scale for MM, MF, FM and FF is on right y-axis.
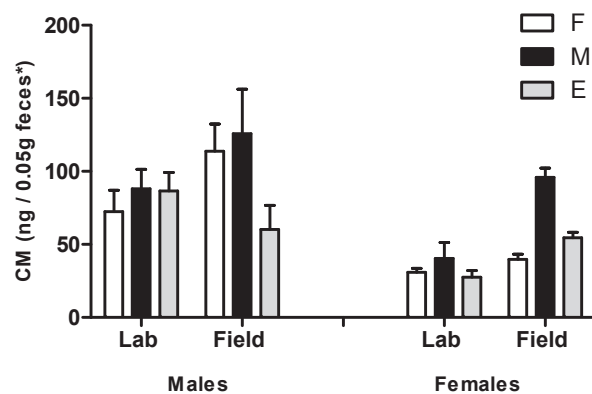
FIGURE 3　　Fecal corticosterone metabolite levels (LS-means with SE) indicating stress in different population types in females and males. In the laboratory (Lab) all animals were housed individually. (F = female biased population type, M = male biased population type, E = even sex-ratio population type, * to enable accurate comparison between sexes the metabolite levels are adjusted. See Material and methods, *Fecal sampling and analyses*).
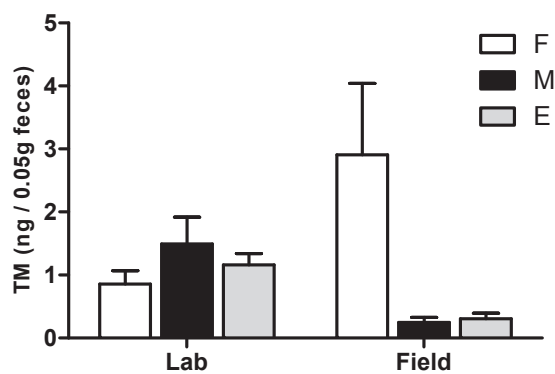
FIGURE 4　　Fecal testosterone metabolite (TM) levels (LS-means with SE) in different population types in males. In the laboratory (Lab) all animals were housed individually. (F = female biased population type, M = male biased population type, E = even sex-ratio population type).

II

CHANGING WINTER CONDITIONS IN THE BOREAL FOREST: THE EFFECTS OF FLUCTUATING TEMPERATURE AND PREDATION RISK ON ACTIVITY AND STRESS IN BANK VOLES

by

Saana Sipari, Marko Haapakoski, Ines Klemme, Rupert Palme, Janne Sundell & Hannu Ylönen 2014

Manuscript

CHANGING WINTER CONDITIONS IN THE BOREAL FOREST: THE EFFECTS OF FLUCTUATING TEMPERATURE AND PREDATION RISK ON ACTIVITY AND STRESS IN BANK VOLES

Saana Sipari[1], Marko Haapakoski[1], Ines Klemme[1], Rupert Palme[3], Janne Sundell[2], and Hannu Ylönen[1]

1 Department of Biological and Environmental Science, Konnevesi Research Station, University of Jyväskylä, P.O.Box 35, FI-40014 Jyväskylä, Finland; 2 Lammi Biological Station, University of Helsinki, Pääjärventie 320, 16900 Lammi, Finland; 3 Institute of Physiology, Pathophysiology and Biophysics, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

ABSTRACT

Due to the global climate change the winter conditions in the North are predicted to change, as the time with an intact snow cover gets shorter or disappears altogether. Many small ground dwelling animals, like rodents, are strongly dependent on protection provided by snow cover against cold and predators. Thus, lack of proper snow cover causing exposure to strong temperature fluctuations and increased predation risk could induce severe stress, causing alterations in the physical condition and behavior, and eventually lead to lower winter survival. To test this, we exposed bank voles (*Myodes glareolus*) to different temperature regimes and cues of predator threat under laboratory conditions. The test animals experienced either a stable but cool temperature regime resembling the stable conditions under snow cover, or an unstable diel temperature regime with cold nights and warmer days simulating the climate change scenario with unstable winter. After three weeks, the animals were additionally exposed to owl calls or sounds of non-predatory bird species. Stress levels, activity, food consumption and body mass were monitored. We observed that the voles exposed to unstable temperatures adjusted their normal mostly nocturnal circadian activity pattern towards a more diurnal rhythm without any significant responses in their stress level. Introducing the sound manipulation elevated the stress levels in females but not in males. However, the laboratory born voles were not able to distinguish between the sound of the non-predatory control bird and predatory bird and seemingly considered both as a threat. The sound induced stress levels did not differ between the temperature treatments. However, the temperature regime tended to affect anti-predator behavior as individuals experiencing unstable temperature and a threatening sound decreased their overall activity, unlike individuals under stable temperature treatments. Our results suggest that the ability of bank voles to plastically adjust their behavior may diminish the accumulation of stress when exposed to multiple stressors simultaneously.

Key words: climate change, winter, stress, unstable temperature, predation risk

INTRODUCTION

The recent course of global climate change has raised many questions about its potential consequences on biodiversity and ecosystem functions around the world. Both local and global extinctions are predicted to occur due to the rapidly changing environmental conditions, as in many parts of the world the unpredictable events in climate (e.g. storms, drought) will be more pronounced than before (Lovejoy and Hannah 2005, Stocker *et al.* 2013). To persist in this kind of unstable environment is obviously demanding and requires plasticity from the organisms. The future course of many ecosystems in the northern parts of the world is of particular interest, as climate change is predicted to have notable consequences especially in these areas due to alterations in winter conditions (Moritz *et al.* 2002, Lovejoy and Hannah 2005, Stocker *et al.* 2013).

At high latitudes, snow cover during winter is characteristic and plays an important role in ecosystem dynamics, as many northern animals and plants are strongly dependent on the protection the snow provides against cold and predators (Marchand 1996, Stien *et al.* 2012, Wolkovich *et al.* 2012, Mills *et al.* 2013). However, due to global warming the winters in the North are about to go through some drastic changes as the time period with an intact snow cover gets shorter, or in the worst case, vanishes altogether (Serreze *et al.* 2000, Lovejoy and Hannah 2005, Jylhä *et al.* 2008). For small ground-dwelling mammals the lack of snow would expose them to severe temperature fluctuation and increase their susceptibility to many predators (Marchand 1996). This could lead to strongly elevated stress levels and consequently, deteriorated winter survival.

However, animals are capable of adapting to new environmental conditions but it often requires both behavioral and physiological responses to survive in suddenly deteriorating environmental circumstances (Wingfield and Sapolsky 2003, Boonstra 2004, Wingfield 2013, Boonstra *et al.* 2014). Under unfavorable external conditions animals usually face inevitable trade-offs. Often organisms allocate resources from acutely less vital functions, like reproduction or somatic growth (McNamara and Buchanan 2005, Bronson 2009). For example, under elevated predation pressure animals usually adjust their behavior accordingly, e.g. reducing activity and the time spent for foraging (Preisser *et al.* 2005). This, in the long run, however, can lead to an insufficient energy gain. In the case of abiotic stressors, such as cold stress, maintaining homeostasis by thermoregulation is energy consuming. If the availability and quality of nutrition is poor, it can eventually lead to the neglect of other physiological functions (e.g. immunocompetence) causing even pathological conditions (McEwen and Wingfield 2003, McEwen 2004, Beldomenico *et al.* 2008) When multiple stressful events occur simultaneously the reallocation of resources gets even more demanding (McNamara & Buchanan 2005).

The unpredictable winter conditions and lack of a proper snow cover could expose small boreal mammals to several stressors at the same time. This could be the case especially during the early winter and early spring, as winters, in terms of permanent snow cover, are expected to become shorter in

the future (Moritz *et al.* 2002, Lovejoy and Hannah 2005, Jylhä *et al.* 2008, Stocker *et al.* 2013). Here, we tested the hypothesis that climate change causes both behavioral and physiological responses in a boreal small mammal, the bank vole (*Myodes glareolus*). To mimic the possible effects of climate change we exposed test animals to different temperature regimes and cues of predators. Voles were experiencing either a stable but cool temperature regime resembling the stable conditions under the snow cover, or an unstable diel temperature regime with cold nights and warm days simulating the climate change scenario with unstable winter and early snow melt. In addition, we exposed animals to owl calls representing an elevated predation risk, or sounds of non-predatory bird species. Resident owls are a significant source of predation for small mammals and their activity includes frequent calling during late winter and early spring (Laine 2009, Korpimäki and Hakkarainen 2012). Our hypothesis was that under the climate change conditions without the protective snow cover the animals would be stressed because of the strong fluctuations in temperature and that they would adjust their activity towards a diurnal rhythm to avoid cold nights. We also predicted that unstable temperatures combined with predation threat would accumulate the stress reaction leading to strongly reduced overall activity. Thus, we expected to see the highest stress levels and the lowest activity in treatments with variable temperature and predatory cues. In stable temperature treatments with non-predatory bird sounds the activity was expected to stay species-typical and stress levels low (Eilam *et al.* 1999, Hettena *et al.* 2014).

MATERIAL AND METHODS

Experimental animals

All animals used in this study (40 males and 32 females) were born in the laboratory in April-July 2012 at the Konnevesi research station, Central Finland. To mimic the natural seasonal rhythm in the wild in Central Finland, voles were overwintered in climate chambers at 6 ^{0}C from November 2012 until the beginning of the experiment in April 2013. The light period was set to resemble the average light regime in winter time with 7 hours of light and 17 hours of darkness. As communal nesting is suggested to be common for *Myodes* voles during winter under natural conditions (Merritt 1984, Ylönen and Viitala 1985) we housed the voles in same-sex groups of four individuals in large cages (60×40×20 cm) until February. After that, they were separated in to individual standard mouse cages (43×26×15 cm) to habituate for the experiment. We also gradually increased the day length. All the voles had wood shavings and cotton wool as bedding and food (mice pellets, Labfor R36, Lantmännen) and water were provided *ad libitum*.

At the beginning of April 2013 all voles were weighed and injected with subcutaneous PIT tags for monitoring their activity (see below). After this, voles were introduced to their experimental cages (see below) and divided evenly into four climate chambers according to sex and body mass. In each

chamber we housed 10 males and 8 females, and the mean body mass was approximately the same in all four treatments (mean 22.9 ± 0.35 g, $F_{1,68}$ = 0.188, p = 0.666). Throughout the experiment the light regime of the chambers were set to 12 L: 12 D, resembling the day length of late winter/ early spring in Central Finland. The light regime was constant during the whole experiment.

Experimental cages

To monitor daily activity, standard mouse cages were equipped with a separating wall across the middle of the cage made from plywood and a hole large enough for a vole to go through (Ø 5cm). In 36 (out of 74) of these cages there was a sensor (Trovan®, EID Aalten BV, Aalten, Holland) around the hole connected to a PC, which recorded each passage of the PIT-tagged individual through the hole (date and time) . The activity sensing cages were distributed equally across all treatments (18 for stable temperature chambers and 18 for unstable temperature chambers). The remaining cages had an otherwise similar structure but without the sensor system. Food and water were provided only in one side of the plywood wall, and nest material in the other.

Experimental design

The experiment was carried out in four temperature adjustable laboratories, i.e. climate chambers at the Konnevesi Research Station, Central Finland during April - May 2013. To study the effects of fluctuating temperature and predation risk on activity and stress levels in bank voles, we generated different treatments. Voles were experiencing either a stable but cool temperature mimicking the conditions under intact snow cover in late winter / early spring, or fluctuating daily temperature with cold nights and warm days representing the climate change scenario with unstable snow cover and early melting snow, exposing them to strong fluctuation of ambient temperature. In the stable temperature treatment the temperature was constant at 6˚C (+/- 1˚C), whereas in the unstable temperature treatments it fluctuated from 1˚C at night to 12˚C during the day (+/- 1˚C). Thus, the average daily temperature was approximately the same in both treatments. Under natural conditions the temperature would of course be lower, as the temperature under the snow cover stays generally around 0˚C, and the temperature fluctuations without the snow cover could be more severe. However, we want to emphasize, that the ultimate purpose of the manipulations was to act as an indicator of a stable environment and unstable environment, rather than examine the effect of absolute temperatures *per se*. Temperature manipulation continued through the whole experiment as described.

Predation risk was manipulated using tawny owl (*Strix aluco*) calls played from loudspeakers. Tawny owls are common nocturnal predators in forested areas, often occupying the same habitats as bank voles (Laine 2000). Songs of

the redwing (*Turdus iliacus*), which is a non-predatory early arriving migrant thrush (Laine 2000), was used as a control. Depending on the treatment, either owl calls or thrush sounds were played three times per night (at 21:00, 01:00 and at 05:00) one hour at a time during two nights at standardized sound volume. However, to separate the possible reactions from stress caused by a new stimulus (i.e. new sound) and stress caused by recognized predation threat (owl calls), we performed a preceding sound treatment. One week before the actual predation manipulation, we exposed animals to a mixture of basic forest background sounds, e.g. distant birds, wind, water etc. Sounds of tawny owl (or any predatory species) or redwing were not present in the background recording. The sound regime was the same as above, but instead of two nights it was played for one week during each night.

With these manipulations we generated the following treatments: Unstable temperature with no sound treatment (UT/NS), unstable temperature /thrush song (UT/Control), unstable temperature / owl calls (UT/Owl). Also, stable temperature with no sound treatment (ST/NS), stable temperature / thrush song (ST/Control) and stable temperature / owl calls (ST/Owl). Temperature treatment remained the same for each individual through the whole experiment but sound manipulation alternated, so that each vole experienced both predator sound and control sound. To make the predator and control sound treatments more natural the owl calls and thrush song were recorded on top of slightly muffled background sound. The experiment was carried out according to following time table (Table 1).

Food consumption was measured by weighing the pellets given to the animals and weighing the leftovers. As an indicator of stress we used the level of corticosterone metabolites detected from the feces (Eccard et al 2011; Ylönen et al. 2006; Touma et al 2003; Sipari, Ylönen and Palme, unpublished results). The sound treatments (Owl or Control) were played during the first two nights of weeks 4 and 5. In the morning, after the second night, the pellet consumption was measured and fecal samples collected. The forest background sounds were always played during the rest of the week. Daily activity was monitored and recorded non-stop via the Trovan-system, but for activity analyses we selected 24 h periods from each week, the same for all individuals. At the end of the experiment all animals were weighed again.

Fecal sampling and analysing

To collect fecal samples each vole was temporarily moved to a clean and empty (no beddings) mouse cage. After the vole defecated it was returned to its home cage. This usually happened quickly, max. in one hour. After this, feces were collected to empty Eppendorf-tubes (1.5 ml, one tube per individual per session) from the sampling cage using tweezers. Samples were stored at -20 ˚C. All fecal samples were analyzed by using 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA), with the methods described in Touma et al. 2003. This specific EIA has been validated and proven suitable for measuring fecal

corticosterone metabolites in bank voles (Sipari, Ylönen and Palme, unpublished results). In that particular validation experiment it was shown that males excrete on average 70 % of corticosterone metabolites via feces, whereas females excrete only around 50 %. Due to this significant difference we decided to adjust the measured corticosterone metabolite levels to the estimated value of 100% for both sexes for meaningful comparisons in our statistical analyses and in illustrations. All laboratory analyses were performed in the University of Veterinary Medicine, in Vienna, Austria.

Statistical analyses

Statistical analyses were performed using R 3.0.3 and IBM SPSS Statistics 20. Corticosterone metabolite levels (stress), activity, food consumption and body mass were all analysed using linear mixed model (with Gaussian error structure), fit by REML. Temperature treatment (ST and UT), sound treatment (NS, Control and Owl) and sex were set as fixed factors, and ID and chamber as random factors when analysing stress levels and activity. Stress levels were also tested between the sexes separately (see Material and methods, *Fecal sampling and analysing*). To compare night and day activity within and between groups during different sound treatments, the model was extended by adding time (12D:12L, night = 8 pm - 8 am and day = 8 am - 8 pm) as a fixed factor. To control for playing order of Control and Owl sound we originally included the "order" as a fixed factor to our models. However, this caused strong multicollinearity between variables and we omitted this variable from the model. Instead, we tested the effect of playing order separately for stress levels and activity and found no significant effect. To test the effects of our treatments on body mass we used temperature treatment, time (start and end of the experiment) and sex as fixed factors, and ID and chamber as random factors. Food consumption was tested with temperature, sound treatment and sex as fixed factors, and again, ID and chamber as random factors. Daily food consumption was calculated by dividing the amount of food eaten by the number of days between measurements. For model selection we used the Akaike information criterion (AIC), model with the lowest AIC value selected for the analyses. To test for a correlation between stress level and total activity, and activity and food consumption, we used Spearman's correlation.

RESULTS

Stress

Stress levels measured as corticosteroid metabolites were significantly higher in males than in females ($F_{1,64}$ = 28.565, p < 0.001) throughout the experiment (Fig 1). When the sexes were analyzed separately, stress levels in females were significantly affected by the sound treatment ($F_{2,53}$ = 5.052, p = 0.010) but not by

the temperature treatment ($F_{1,30} = 0.344$, p = 0.562). In a pairwise comparison the stress levels in the Control were significantly higher than in NS ($T_{55.37} = 3.090$, p = 0.009), and the difference between NS and Owl was nearly significant ($T_{55.91} = -2.384$, p = 0.052). There was no significant difference between Control and Owl ($T_{54.51} = 0.674$, p = 0.779). No significant interactions. In males there were no significant differences between any treatments (temperature: $F_{1,34} = 0.398$, p = 0.532, sound: $F_{2,62} = 0.0278$, p = 0.973). However, there was a significant interaction between temperature treatment and sound treatment ($F_{2,62} = 6.889$, p = 0.002), but in pairwise comparisons all differences were not significant (all comparisons p > 0.080).

Activity

Total activity was significantly affected by the sound treatments ($F_{2,119} = 3.7749$, p = 0.026) but not by the temperature treatments ($F_{1,28} = 1.667$, p = 0.207). The interaction of sound and temperature was close to significant ($F_{2,119} = 2.979$, p = 0.055). Activity did not differ significantly between the sexes ($F_{1,28} = 0.902$, p = 0.351). There were different trends in the response to the sound treatments between temperature groups (Fig 2). Individuals in ST treatment tended to increase their activity as a response to sound treatments, whereas individuals from UT decreased their activity. However, in the pairwise comparison only the difference in total activity between NS vs. Owl in UT group reached significance (Z = 3.436, p = 0.008, all other pairwise comparisons > 0.238). We observed no correlation between stress level and total activity (r = 0.116, p = 0.120). In general, individuals in ST treatment were significantly more active during night than day (Z = -8.259, p < 0.001), whereas in UT treatment the difference was not significant (Z = -1.671, p = 0.339). The day time activity in ST treatment was significantly lower than in UT treatments (Z = -3.136, p = 0.009). No significant difference in night time activity between temperature treatments was observed (Z = 0.715, p = 0.891). There were several significant interactions (temperature and time, sound and time, sex and time, and sound and sex; all p < 0.025).

Body mass and food consumption

Males were heavier than females throughout the experiment ($F_{1,67} = 59.646$, p <0.001). Also time had an effect on body mass, as the mean body mass was significantly lower at the beginning of the experiment than in the end ($F_{1,56} = 31.888$ p < 0.001). There were no significant differences between temperature groups ($F_{1,67} = 0.067$, p = 0.796), and no significant interactions.

Food consumption was significantly affected by the sound treatments ($F_{2,126} = 43.372$, p = 0.001) but not by temperature treatments ($F_{1,64} = 1.232$, p = 0.271). Food consumption was significantly lower in NS than Control and Owl (NS vs. Control: Z = -7.613, p < 0.001, NS vs Owl Z = -8.441, p < 0.001). There

was no difference in food consumption between Control and Owl treatments (Z = -0.823, p = 0.689). The increase in food consumption was, however, more likely time dependent rather than a consequence of the sound treatments. In a previous experiment conducted in the same climate chambers at the same time of the year (Sipari et al. 2014) an identical pattern in food consumption was observed without stressors other than temperature regimes. Males tended to eat more than females but the difference was not significant ($F_{1,64}$ = 3.834, p = 0.055). There was no correlation between activity and food consumption (r = 0.136, p = 0.218).

DISCUSSION

Our results suggest that behavioral plasticity may diminish the accumulation of stress in bank voles when exposed to multiple stressors simultaneously. Voles in different temperature regimes did not show differences in stress levels, but individuals experiencing an unstable temperature regime with cold nights and warm days adjusted their activity towards a more diurnal rhythm compared to individuals under stable temperature, who remained species specifically more nocturnal. Both predator and control sound manipulations elevated stress levels in females, but in males no significant response was observed. We suggest that due to the laboratory origin, the voles used in the experiment did not distinguish the level of threat between the sound manipulations, but rather considered both of these sounds potentially threatening. However, we observed divergent trends in the behavioral response to the sound treatments under different temperature regimes. Voles exposed to unstable temperature conditions reduced their activity when encountering a threatening sound, whereas under stable temperature this reaction was not observed. Males had significantly higher stress levels than females throughout the experiment but the difference can be explained by their naturally higher basal level (Sipari, Palme and Ylönen, unpublished results).

Winter in boreal and polar regions is the most challenging season for most species. Thermoregulation is a physiological constraint and a stressor to animals experiencing temperatures far below their so called lower critical temperature (LCT). Lower critical temperature is the ambient temperature where an animal needs to adjust its behavior and physiological functions (e.g. increased/reduced mobility, energy intake, shivering) in order to maintain homeostasis (Marchand 1996). LCT is often season and acclimatization dependent (McDevitt and Speakman 1994, Marchand 1996). Exposure to cold increases the metabolic rate in non-hibernating small mammals (Wang *et al.* 1999, Cichon *et al.* 2002) and it decreases immunocompetence (Cichon *et al.* 2002). We hypothesized that compared to cool but stable temperature (6^0C), fluctuating cool temperatures (1^0C - 12^0C) could be even more challenging for small animals, due to the constant adjustment in thermoregulation it requires. However, we did not find significantly elevated stress levels in unstable temperature treatment, unlike expected. This result could indicate that voles

acclimatized to cool temperature (see Material and methods) are relatively adaptable to changes in temperature, at least in the case of unrestricted food availability. Further, we observed a clear plasticity in their circadian activity patterns, which likely plays an important role in their successful adaptation to different temperature conditions. In bank voles the circadian rhythm in activity is polyphasic (Ylönen 1988, Halle 2000). Instead of one continuous phase of activity and rest bank voles have several activity bouts separated with resting periods during 24 hours periods. The majority of these activity bouts occur between dusk and dawn, while daytime activity is often relatively low (Ylönen 1988). However, as predicted, animals in unstable temperature treatments experiencing cold nights and warmer days increased their diurnal activity compared to individuals under stable temperature. Total activity did not differ between temperature treatments. Thus, our results suggest that bank voles are able to adjust their circadian activity patterns according to their environment, without any obvious stress reaction.

Introducing the sound treatments caused significantly elevated corticosterone metabolite levels in females but did not differ between the experienced temperature treatments. This implies, that due to the behavioral adjustments, e.g. shift in their circadian activity pattern, the fluctuating temperature combined with predation risk did not induce higher stress levels compared to the stable temperature treatments. The inability to recognize neutral sounds from predator sounds (also the thrush song caused significantly elevated corticosterone metabolite levels) may be explained by the laboratory origin of the voles. In males there were no significant differences in stress levels between any treatments. However, corticosterone levels as indicators of stress in males may be perhaps somewhat more complicated compared to females, as testosterone has been shown to strongly impact glucocorticoids and fear. It is generally accepted that stress reduces testosterone level (Chichinadze and Chichinadze 2008, Bronson 2009), but there is also strong evidence that animals with high testosterone level have lower stress hormone levels and are less fearful to novel stimuli (Cornwell-Jones and Kovanic 1981, Boissy and Bouissou 1994, Place and Kenagy 2000, Schradin 2008, Kohlhause *et al.* 2011). These results could explain some of the variance in stress levels between males in our experiment, especially in the unstable temperature treatments, as our previous experiment showed that variable spring temperature induces testosterone production in bank vole males (Sipari *et al.* 2014). In addition, despite the short day rhythm the animals experienced before the experiment, many of the individuals had external indications of sexual maturity already in February. This was unexpected and probably due to the laboratory origin of the voles. Also abundant food availability likely enhanced the maintenance of maturity.

Despite the lack of response on stress levels, the temperature treatments seemed to affect the behavioral response of bank voles when encountering a threatening sound. Voles exposed to unstable temperature conditions significantly reduced their activity in respond to the sound treatment, whereas voles under stable temperature rather increased their activity, though not

significantly. This may indicate that abiotic environmental conditions may affect anti-predator behavior in bank voles. However, we should point out that the voles within a temperature group showed no statistically significant difference in activity when comparing their responses between the thrush sound and owl sound, only against treatment with no sound at all. That is why the term "anti-predator" behavior should be interpreted cautiously. Nevertheless, we consider using the term in this context justified, as based on our results it seems that both of these sounds were interpreted as a threat.

In prey animals, like voles, the anti-predator behavior is often divided in two main responses; fleeing and freezing. Based on the trend observed in our experiment it seems that the fleeing response was more pronounced under stable temperatures, whereas under unstable temperatures reduced activity was more common reaction. Anti-predator behavior can differ according to predator type (terrestrial or avian), distance to predator and behavior of the predator (immobile or approaching etc.) or whether the threat is direct or indirect (Ydenberg and Dill 1986, Eilam *et al.* 1999, Edut and Eilam 2004, Stankowich and Blumstein 2005, Blumstein 2006, Cooper *et al.* 2012). Also the geography of a habitat (open or covered) makes a difference (Jacob and Brown 2000). There are also indications that the reproductive stage and life history of an animal can affect anti-predator behavior (Borowski 2002, Brown and Shine 2004, Trebatická *et al.* 2010). Our results suggest that perhaps also the abiotic environmental conditions, such as climatic or weather conditions, could affect the chosen anti-predator strategy. However, the reason for the different trends in the behavior between temperature treatments observed in our experiment is still unclear. Banks et al. 2002 showed that, due to the innate fear response, unfamiliar habitat increases immobility in *Microtus* voles. Perhaps one could argue that an unstable abiotic environment could act as some kind of unpredictability factor comparable to the unfamiliar habitat in the study of Banks et al. 2002.

Laboratory experiments often provide only a coarse indication and idea of the ecological interactions occurring under natural conditions. As anti-predator behavior is a summary of multiple factors, extrapolating our results to natural conditions as such is not possible. However, when speculating the feasibility of the observed responses, one could argue that the shift in the circadian activity pattern as well as reduced activity under predation risk observed in individuals in unstable temperature could be considered logical reaction under the conditions of the climate change scenario. Owls prey mainly based on their vision and hearing, thus, prey living in habitat with little shelter, e.g. no snow cover, would probably survive better by reducing activity. However, under long term predation risk the reduced activity could lead to energy deficiency if the food availability is scarce, as often is the case during winter. In our experiment there was no difference in food consumption nor body mass between temperature groups, despite the opposite activity trends between groups. This is probably due to the abundance and vicinity of the food source. Further, in the wild reduced mobility would eventually also increase the accumulation of scent cues (Banks *et al.* 2000), and likely increase the risk of

mammalian scent-based predators. The shift in their circadian rhythm towards more diurnal activity is a logical response against nocturnal predators, as well as against cold nights, but could again increase the likelihood of being preyed on by day active predators. Nevertheless, these results provide an interesting aspect to the study of anti-predator behavior by suggesting that abiotic environmental factors might affect the anti-predator strategy in certain species.

Conclusion

Our results suggest that changes in winter conditions caused by the climate change may alter the circadian activity pattern and possibly even the anti-predator behavior of bank voles. However, the adaptability and behavioral plasticity in bank voles may diminish the risk of pathological accumulation of stress effects in cases of multiple simultaneous stressors. On the other hand, not all stressors are equal. In addition to the suggested increase in winter time predation pressure and temperature related stress in small mammals, the food availability is suggested to become compromised. Thus, in future studies it would be interesting to test how the food limitation combined with different temperature or predation stressors would affect the behavioral and physiological responses, and potentially the winter survival in bank voles.

REFERENCES

Banks P.B., Norrdahl K. & Korpimäki E. 2000. Nonlinearity in the predation risk of prey mobility. *Proc. Biol. Sci.* 267: 1621-1625.

Beldomenico P.M., Telfer S., Gebert S., Lukomski L., Bennett M. & Begon M. 2008. Poor condition and infection: a vicious circle in natural populations. *Proc. Biol. Sci.* 275: 1753-1759.

Blumstein D.T. 2006. Developing an evolutionary ecology of fear: how life history and natural history traits affect disturbance tolerance in birds. *Anim. Behav.* 71: 389-399.

Boissy A. & Bouissou M.F. 1994. Effects of androgen treatment on behavioral and physiological responses of heifers to fear-eliciting situations. *Horm. Behav.* 28: 66-83.

Boonstra R. 2004. Coping with changing northern environments: the role of the stress axis in birds and mammals. *Integr. Comp. Biol.* 44: 95-108.

Boonstra R., Dantzer B., Delehanty B., Fletcher Q.E. & Sheriff M.J. 2014. Equipped for Life in the Boreal Forest: The Role of the Stress Axis in Mammals. *Arctic* 67: 82-97.

Borowski Z. 2002. Individual and seasonal differences in antipredatory behaviour of root voles a field experiment. *Can. J. Zool.* 80: 1520-1525.

Bronson F.H. 2009. Climate change and seasonal reproduction in mammals. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 364: 3331-3340.

Brown G. & Shine R. 2004. Effects of reproduction on the antipredator tactics of snakes (Tropidonophis mairii, Colubridae). *Behav. Ecol. Sociobiol.* 56: 257-262.

Chichinadze K. & Chichinadze N. 2008. Stress-induced increase of testosterone: Contributions of social status and sympathetic reactivity. *Physiol. Behav.* 94: 595-603.

Cichon M., Chadzinska M., Ksiazek A. & Konarzewski M. 2002. Delayed effects of cold stress on immune response in laboratory mice. *Proc. Biol. Sci.* 269: 1493-1497.

Cooper W.E., López P., Martín J. & Pérez-Mellado V. 2012. Latency to flee from an immobile predator: effects of predation risk and cost of immobility for the prey. *Behav. Ecol.* 23: 790-797.

Cornwell-Jones C.A. & Kovanic K. 1981. Testosterone reduces olfactory neophobia in male golden hamsters. *Physiol. Behav.* 26: 973-977.

Eccard J.A., Fey K., Caspers B.A. & Ylonen H. 2011. Breeding state and season affect interspecific interaction types: indirect resource competition and direct interference. *Oecologia* 167: 1-11.

Edut S. & Eilam D. 2004. Protean behavior under barn-owl attack: voles alternate between freezing and fleeing and spiny mice flee in alternating patterns. *Behav. Brain. Res.* 155: 207-216.

Eilam D., Dayan T., Ben-Eliyahu S., Schulman I., Shefer G. & Hendrie C.A. 1999. Differential behavioural and hormonal responses of voles and spiny mice to owl calls. *Anim. Behav.* 58: 1085-1093.

Halle S. 2000. Voles—small graminivores with polyphasic patterns. In: Anonymous Activity patterns in small mammals, Springer, pp. 191-215.

Hettena A.M., Munoz N. & Blumstein D.T. 2014. Prey Responses to Predator's Sounds: A Review and Empirical Study. *Ethology* 120: 427-452.

Jacob J. & Brown J.S. 2000. Microhabitat use, giving-up densities and temporal activity as short- and long-term anti-predator behaviors in common voles. *Oikos* 91: 131-138.

Jylhä K., Fronzek S., Tuomenvirta H., Carter T.R. & Ruosteenoja K. 2008. Changes in frost, snow and Baltic sea ice by the end of the twenty-first century based on climate model projections for Europe. *Clim. Change* 86: 441-462.

Kohlhause S., Hoffmann K., Schlumbohm C., Fuchs E. & Flügge G. 2011. Nocturnal hyperthermia induced by social stress in male tree shrews: relation to low testosterone and effects of age. *Physiol. Behav.* 104: 786-795.

Korpimäki E. & Hakkarainen H. 2012. *The Boreal Owl: ecology, behaviour and conservation of a forest-dwelling predator.* Cambridge University Press.

Laine L.J. 2009. *Suomen luonto-opas.* WSOY.

Lovejoy T.E. & Hannah L.J. 2005. *Climate change and biodiversity / edited by Thomas E. Lovejoy & Lee Hannah.* Yale University Press.

Marchand P.J. 1996. *Life in the cold: an introduction to winter ecology.* University Press of New England.

McDevitt R.M. & Speakman J.R. 1994. Limits to sustainable metabolic rate during transient exposure to low temperatures in short-tailed field voles (Microtus agrestis). *Physiol. Zool.* 67: 1103-1116.

McEwen B.S. 2004. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Ann. N. Y. Acad. Sci.* 1032: 1-7.

McEwen B.S. & Wingfield J.C. 2003. The concept of allostasis in biology and biomedicine. *Horm. Behav.* 43: 2-15.

McNamara J. & Buchanan K. 2005. Stress, resource allocation, and mortality. *Behav. Ecol.* 16: 1008-1017.

Merritt J.F. 1984. *Winter ecology of small mammals.* Carnegie Museum of Natural History, Pittsburgh.

Mills L.S., Zimova M., Oyler J., Running S., Abatzoglou J.T. & Lukacs P.M. 2013. Camouflage mismatch in seasonal coat color due to decreased snow duration. *Proc. Natl. Acad. Sci. U S A* 110: 7360-7365.

Moritz R.E., Bitz C.M. & Steig E.J. 2002. Dynamics of recent climate change in the Arctic. *Science* 297: 1497-1502.

Place N. & Kenagy G. 2000. Seasonal changes in plasma testosterone and glucocorticosteroids in free-living male yellow-pine chipmunks and the response to capture and handling. *J. Comp. Physiol. B.* 170: 245-251.

Preisser E.L., Bolnick D.I. & Benard M.F. 2005. Scared to death? The effects of intimidation and consumption in predator-prey interactions. *Ecology* 86: 501-509.

Schradin C. 2008. Seasonal changes in testosterone and corticosterone levels in four social classes of a desert dwelling sociable rodent. *Horm. Behav.* 53: 573-579.

Serreze M., Walsh J., Chapin Iii F., Osterkamp T., Dyurgerov M., Romanovsky V., Oechel W., Morison J., Zhang T. & Barry R. 2000. Observational evidence of recent change in the northern high-latitude environment. *Clim. Change* 46: 159-207.

Sipari S., Haapakoski M., Klemme I., Sundell J. & Ylönen H. 2014. Sex-specific variation in the onset of reproduction and reproductive trade-offs in a boreal small mammal. *Ecology* 95: 2851-2859.

Stankowich T. & Blumstein D.T. 2005. Fear in animals: a meta-analysis and review of risk assessment. *Proc. Biol. Sci.* 272: 2627-2634.

Stien A., Ims R.A., Albon S.D., Fuglei E., Irvine R.J., Ropstad E., Halvorsen O., Langvatn R., Loe L.E., Veiberg V. & Yoccoz N.G. 2012. Congruent responses to weather variability in high arctic herbivores. *Biology letters* 8: 1002-1005.

Stocker T.F., Qin D., Plattner G., Tignor M., Allen S.K., Boschung J., Nauels A., Xia Y., Bex V. & Midgley P.M. 2013. Climate change 2013: The physical science basis. *Intergovernmental Panel on Climate Change, Working Group I Contribution to the IPCC Fifth Assessment Report (AR5)(Cambridge Univ Press, New York)* .

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

Trebatická L., Ketola T., Haapakoski M., Opperbeck A. & Ylönen H. 2010. Is Fear a Factor in Voles? Short Term Physiological and Behavioural Response to Predation Stress. *Open Ecology Journal* 3: 16-21.

Wang D., Sun R., Wang Z. & Liu J. 1999. Effects of temperature and photoperiod on thermogenesis in plateau pikas (Ochotona curzoniae) and root voles (Microtus oeconomus). *J. Comp. Physiol. B.* 169: 77-83.

Wingfield J. & Sapolsky R. 2003. Reproduction and resistance to stress: when and how. *J. Neuroendocrinol.* 15: 711-724.

Wingfield J.C. 2013. The comparative biology of environmental stress: behavioural endocrinology and variation in ability to cope with novel, changing environments. *Anim. Behav.* 85: 1127-1133.

Wolkovich E.M., Cook B.I., Allen J.M., Crimmins T.M., Betancourt J.L., Travers S.E., Pau S., Regetz J., Davies T.J., Kraft N.J.B., Ault T.R., Bolmgren K., Mazer S.J., McCabe G.J., McGill B.J., Parmesan C., Salamin N., Schwartz M.D. & Cleland E.E. 2012. Warming experiments underpredict plant phenological responses to climate change. *Nature* 485: 494-7.

Ydenberg R.C. & Dill L.M. 1986. The economics of fleeing from predators. *Adv. Study Behav.* 16: 229-249.

Ylönen H. 1988. Diel activity and demography in an enclosed population of the vole Clethrionomys glareolus(Schreb.). *Ann. Zool. Fennici* 25: 221-228.

Ylönen H. & Viitala J. 1985. Social organization of an enclosed winter population of the bank vole Clethrionomys glareolus. *Ann. Zool. Fennici* 22: 353-358.

Ylönen H., Eccard J.A., Jokinen I. & Sundell J. 2006. Is the antipredatory response in behaviour reflected in stress measured in faecal corticosteroids in a small rodent? *Behav. Ecol. Sociobiol.* 60: 350-358.

TABLE 1. The time table of the experiment

| Time	Treatment	Experimental procedures
Week 1	Temperature (ST, UT), no sound	- no procedures, habituation period
Week 2	Temperature (ST, UT), no sound	- monitoring daily activity - measuring food consumption - collecting fecal samples
Week 3	Temperature + Back ground sound	- no procedures, habituation for sound
Week 4	Temperature + Owl or Control sound	- monitoring daily activity - measuring food consumption - collecting fecal samples
Week 5	Temperature + Owl or Control sound (reversed)	- monitoring daily activity - measuring food consumption - collecting fecal samples
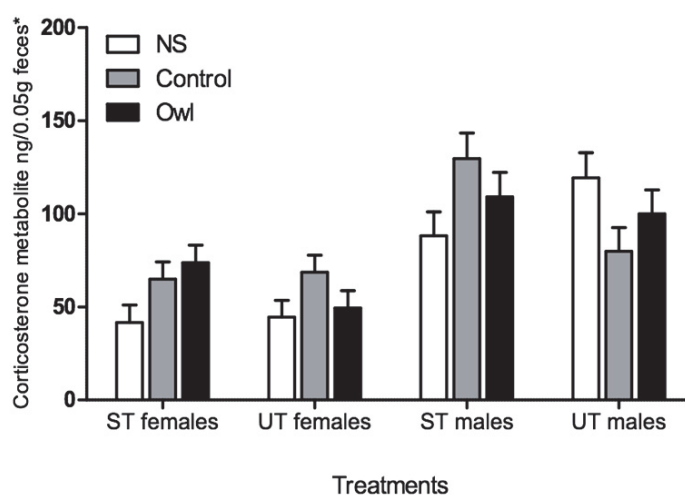
FIGURE 1   LS-means (with SE) of corticosterone metabolite levels indicating stress in different treatments for females and males. ST = stable temperature treatment, UT = unstable temperature treatment. NS = no sound, Control = control sound and Owl = predator sound (* To enable accurate comparison between sexes the metabolite levels are adjusted. See Material and methods).
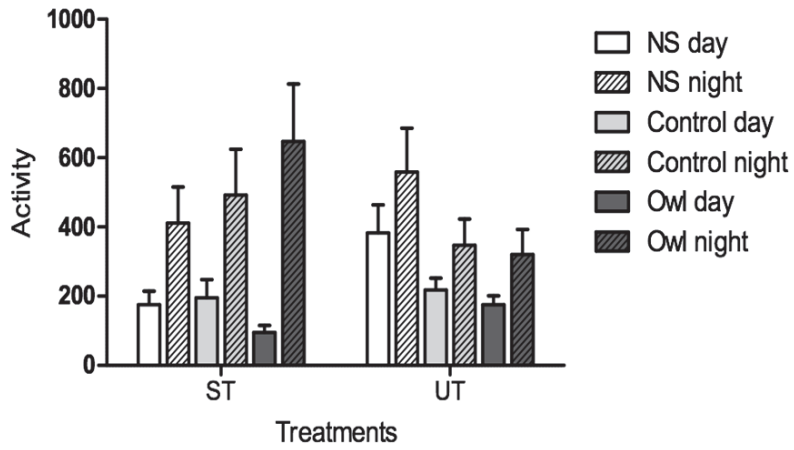
FIGURE 2   LS-mean (with SE) activity during day and night in different treatments. Activity on x-axis represents the frequency of recorded entries through the sensor hole in the experimental cages during 24 hours (see Material and methods, *Experimental cages*). ST = stable temperature treatment, UT = unstable temperature treatment, NS = no sound, Control = control sound and Owl = predator sound.

III

SEX SPECIFIC VARIATION IN THE ONSET OF REPRODUCTION AND REPRODUCTIVE TRADE-OFFS IN A BOREAL SMALL MAMMAL

by

Saana Sipari, Marko Haapakoski, Ines Klemme, Janne Sundell & Hannu Ylönen 2014

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Sex-specific variation in the onset of reproduction and reproductive trade-offs in a boreal small mammal

Saana Sipari,[1,3] Marko Haapakoski,[1] Ines Klemme,[1] Janne Sundell,[2] and Hannu Ylönen[1]

[1]*Department of Biological and Environmental Science, Konnevesi Research Station, University of Jyväskylä, P.O. Box 35, FI-40014 Jyväskylä, Finland*
[2]*Lammi Biological Station, University of Helsinki, Pääjärventie 320, 16900 Lammi, Finland*

Abstract. In seasonal environments, the optimal onset of reproduction plays a major role in defining the reproductive success of an individual. Environmental cues, like day length, weather conditions, and food, regulate the initiation and termination of the breeding season. Besides the interspecific variation in response to environmental cues, it has been suggested that due to different selection pressures, females and males can have different responses to environmental stimuli. However, this phenomenon has gained relatively little consideration, and the physiological mechanism behind these differences is not well known. Here, we report how two different environmental cues, variability of temperature and nutritional conditions in spring, affect the onset of breeding in a boreal small rodent, the bank vole, *Myodes glareolus*. We exposed wild-trapped individuals to four different treatments manipulating temperature (stable vs. variable) and food quality (high vs. low protein content) over five weeks in the laboratory. We monitored body-mass development, food consumption, and initiation of breeding. We found sex-specific responses to temperature variability, as males achieved their breeding condition faster in variable temperature treatments, whereas female maturation was delayed. Food quality had no effect on the onset of breeding. To test for possible reproductive trade-offs caused by reproductive decisions made in early spring, the experiment was continued in large outdoor enclosures. There seemed to be no significant long-term effects on reproduction, but early summer survival was affected by climate conditions experienced in spring. Our results show clear sex differences in the response to environmental cues regulating the onset of reproduction in bank voles. Hence, our study suggests that when an organism experiences rapid environmental changes, as are occurring on a global scale, divergent cues may lead to a reproductive mismatch between the sexes. This could noticeably decrease the fitness of many seasonally breeding species.

Key words: environmental cues; food quality; Myodes glareolus; *onset of reproduction; reproductive trade-offs; sex-specific variation; variable temperature.*

INTRODUCTION

Seasonality plays a major role in the life history of many organisms. It sets the rhythm of life cycles of individuals and shapes population dynamics. In particular, seasonality has a strong effect on breeding, as it determines the suitable period for reproduction. In temperate and polar regions, breeding usually occurs during spring and summer, whereas in autumn, the change in light regime triggers physiological changes leading to the cessation of sexual activity in most animals (Marchand 1996). In winter, organisms generally remain in a non-reproductive stage due to limited resources and adverse weather conditions. Thus, with increasing latitude, the breeding season becomes shorter, and at the same time it becomes more important to time the onset of reproduction in order to maximize the reproductive output.

Migratory birds are probably the most widely used species in studies investigating the optimal onset of breeding in seasonal environments (Drent and Daan 1980, Kokko 1999, Bêty et al. 2003, Dunn 2004). Many migratory birds time their migration and arrival to breeding grounds so that hatching occurs near the peak of food abundance when the energetic demands are the highest (reviewed by Daan et al. 1988). Further, the early breeders seem to have the highest reproductive success in the form of the best territories and greater competitive ability of early-born young (Perrins 1970, Nilsson 1989, Norris 1993). However, as a reproductive trade-off, early breeding in unfavorable conditions can be very detrimental. A premature start can be fatal for the offspring, causing large amounts of energy and effort to be wasted, but it can also be fatal for the breeder itself (Ankney and MacInnes 1978, Skinner et al. 1998; in mammals Fairbairn 1977). Not only migratory birds, but also many resident overwintering species like tits, sparrows, woodpeckers, and grouses, as well as small mammals like voles, mice, and squirrels face the same

challenge after the long non-reproductive phase in winter and need to adjust their initiation of breeding carefully (Van Noordwijk et al. 1995). To find the optimal time for the onset of reproduction, animals follow environmental cues, such as photoperiod, food, and temperature (Futuyma 2005).

Photoperiod is the most reliable signal indicating the upcoming breeding season (Gwinner 1986, Hahn et al. 1997), but the abundance and quality of food is one of the most important factors enabling successful reproduction (Karasov and del Rio 2007). Only when the energetic needs for maintaining essential body functions are satisfied can the organism allocate energy for breeding (Bronson 2009). In higher and colder latitudes, temperature sets thresholds for the onset of breeding in many species (Millar and Gyug 1981, Visser et al. 2009). Ambient temperature can be an important factor per se, affecting energetic demands and even gonadal growth in some species (Jones 1986, Meijer et al. 1999). At the same time, temperature contributes to the emergence of fresh food after winter. Hence, food availability and temperature as reproductive indicators are not exclusive but are likely to act together.

Species react differently to environmental cues depending on their physiology, habitat, and behavior (Karasov and del Rio 2007). However, males and females of most species differ significantly in their cost of reproduction (Trivers 1972). Thus, we might expect sex-specific variation in the response to environmental cues. Females typically face higher costs of reproduction than males and are known to use a more extensive variety of environmental cues in a more sophisticated manner than males (reviewed in Ball and Ketterson 2008). A particularly strong imbalance in the cost of reproduction exists among many mammals. In small boreal rodents, females establish and defend a breeding territory in addition to incurring the energetic costs of pregnancy and lactation (Bondrup-Nielsen and Ims 1986, Bujalska 1990, Robbins 1993, Trebaticka et al. 2007). In contrast, males provide nothing but sperm for the offspring. For males, body condition is the major breeding constraint, because it influences gonadal growth and spermatogenesis. In addition, energy is also required for competing with other males and searching for females.

However, both females and males carry the cost of a mistimed onset of breeding. In small rodents, early-breeding females get the advantage of choosing the best breeding territories and have better chances for multiple breeding during the season. Early breeding also enables the offspring to mature in their season of birth, while they can still have access to open breeding territories. On the other hand, breeding too early in unfavorable conditions may lead to nest failure (Millar and Gyug 1981, Sharpe and Millar 1991). To be ready in time for reproduction, many rodent males attain physiological breeding condition slightly earlier than females (Kaikusalo 1972). An early start secures access to females first,

but maintaining high testosterone levels and actively searching for females consumes energy and also exposes the individual to predators (Mills et al. 2009). In contrast to birds, these kind of trade-offs are rarely studied in mammals (but see Fairbairn 1977, Millar and Gyug 1981, Sharpe and Millar 1991) because identifying the onset of breeding can only be assessed from frequent trappings, which are logistically difficult (but see Eccard and Ylönen 2006), and most likely interfere with social interactions, and thus may affect the onset of breeding.

To identify possible sex-specific differences in response to environmental cues and the forms of trade-off, we conducted a combined laboratory and field experiment using a common boreal rodent, the bank vole (*Myodes glareolus*). We studied the onset of breeding under early spring conditions vs. late spring conditions and its fitness consequences for females and males. We manipulated food quality and temperature stability in a 2×2 factorial design over five weeks in a controlled laboratory environment using wild-caught overwintered bank voles just before the onset of breeding. We predicted that females, who carry the higher costs of reproduction, would react more cautiously to environmental cues and initiate breeding earlier in the stable temperature (late spring) and high-quality food treatment. As males provide no parental care, the indirect effect of unstable weather conditions (e.g., nest failure) is more pronouncedly reflected on females. Thus, males were expected to respond more strongly to the quality of food than the temperature regime.

After the laboratory phase, individuals were introduced to large outdoor enclosures for two weeks, and their reproduction and survival were monitored to study long-term fitness effects of spring conditions experienced at the onset of breeding.

Materials and Methods

Experimental animals

Bank voles are common, non-hibernating, omnivorous small rodents, which breed multiple times during the breeding season (see Plate 1). In boreal latitudes, the breeding season is relatively short and usually occurs from April to September (Kaikusalo 1972, Koivula et al. 2003). All animals used in this study (42 males and 34 females) were captured from the wild near the Konnevesi Research Station, central Finland (62°37′ N, 26°20′ E) in early spring (25 March through 14 April 2011) before their breeding season started. For trapping, we used multiple-capture live traps (Ugglan Special, Grahnab, Hillerstorp, Sweden) with sunflower seeds as bait. All individuals were sexually inactive, which was confirmed by observing the vaginal opening (closed) in females and the position of testes (no scrotal testes visible) in males. Experimental animals were marked with ear tags. During the pre-experimental phase, the animals were housed individually in standard mouse cages ($43 \times 26 \times 15$ cm) with sawdust and cotton wool as bedding for 3–20 d at $5° \pm 1°C$ before the actual

treatments started. Food (low-protein pellets, see *Food treatment*) and water were available ad libitum. The photoperiod was 11 h light and 13 h darkness (L:D = 11:13), resembling the light regime of late winter/early spring and thus ensuring that no light-induced sexual maturation would occur before the experimental manipulation commenced.

The experiment consisted of a laboratory phase, conducted in climate chambers and a field phase, conducted in large outdoor enclosures.

Climate chamber phase

The first phase of the experiment was conducted in two temperature-adjustable climate chambers in April–May 2011. The experiment consisted of four treatments manipulating the temperature and food for five weeks.

Temperature treatment.—During the climate chamber phase, the animals experienced either a stable temperature (ST) or variable temperature (VT) treatment (Fig. 1). The stable temperature treatment represents the thermally more stable and predictable conditions the animals would encounter later in spring. In this treatment, the initial temperature was 6°C, and increased slowly to 10°C during the course of the experiment as spring advanced. The variable temperature treatment represents the unstable weather conditions in early spring, when temperature fluctuation is still strong. In the VT treatment, diel temperature in the climate chamber fluctuated from initially 1°C night temperature to 12°C day temperature. These temperatures also increased slightly over the course of the experiment, and at the end of the experiment, the night temperature reached 5°C and the day temperature 16°C. Thus, the average daily temperature was the same in both treatments (increasing from 6° to 10°C ± 1°C).

Naturally, experiments conducted under laboratory conditions have their limitations compared to natural systems. Climatic conditions are hardly ever totally constant in nature. However, the ultimate purpose of the manipulations was to act as an indicator of a stable environment and unstable environment. The mean temperature of both treatments was kept the same in order to keep the experiment's focus on variable (unpredictable early spring) vs. stable (predictable late spring) temperatures, and not to be confounded by the difference in the mean temperatures.

Food treatment.—Animals were provided either with high- (HF) or low- (LF) quality food. Voles in the high-quality food treatment were fed with high-protein pellets (protein content 30%; Altromin Spezialfutter GmbH, Lage, Germany), whereas low-quality food consisted of low-protein pellets (protein content 8%; Altromin Spezialfutter GmbH). All pellets contained an extensive variety of different amino acids, including amino acids normally found both in plant and animal proteins. Only the amount of total protein in the pellets varied between treatments; the total amount of energy between high-quality and low-quality food was the same. High-protein
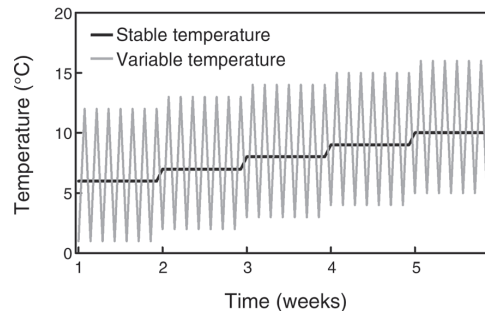

FIG. 1. Temperature patterns in stable and variable temperature treatments during the chamber phase of the study.

pellets represented a high-quality food, as animal protein supplementation studies have shown that increased protein availability advances maturation and reproductive success in females in boreal rodents (McAdam and Millar 1999, Von Blanckenhagen et al. 2007). Differentiation between high and low protein levels was done based on studies of meadow vole (*Microtus pennsylvanicus*) diet, (Lindroth and Batzli 1984, Bergeron and Jodoin 1987, Bucyanayandi and Bergeron 1990), which showed that a protein content under 10% (around 6% in Lindroth and Batzli [1984]) represents protein values in poor habitat (mainly grass and hay). On the other hand, a protein content of 20% and above was found in preferable habitats, often near cultivated areas. Studies that used a similar manipulation of protein content showed that male meadow voles fed with high-protein diets are more attractive to females (Ferkin et al. 1997), and further, dietary protein increases males' sexual signaling with scent marks (Hobbs and Ferkin 2011). Meadow voles are considered to be more herbivorous than bank voles (Butet and Delettre 2011), but for the most part their diets are very similar. In natural conditions, bank voles are highly omnivorous, and the habitat quite strongly dictates the composition of the bank vole diet (Gebczynska 1983, Butet and Delettre 2011). However, if available, bank voles prefer many kinds of seeds and also animal protein (e.g., insects; Gebczynska 1983, Eccard and Ylönen 2006, Butet and Delettre 2011). Both high- and low-quality food was provided ad libitum.

By combining the temperature and food manipulations in a factorial design, we created four experimental treatments: ST, HF; ST, LF; VT, HF; and VT, LF, with ~20 voles in each treatment group. Females and males of different body mass were equally distributed among all treatments, so that the mean mass of each group was approximately the same ($F_{1,75} = 0.013$, $P = 0.998$). The light rhythm in the chambers followed the natural rhythm of spring in central Finland with 14 h of daylight at the beginning of the experiment and 18 at the end. Individuals of the same temperature treatment (ST or VT) were housed in the same climate chamber. All

animals were housed individually (standard mouse cages with sawdust and cotton wool as bedding) except during pairing. During the experiment we monitored (1) food consumption, (2) body-mass development, (3) breeding condition (testosterone levels in males and vaginal opening in females), and (4) onset of breeding. Voles were paired after one week of acclimatization to the treatments by transferring the female into the male's cage. The couples remained paired in the same cage for 15 days. After the separation each vole was again housed individually. Because of an unequal number of males and females the few surplus males were not paired. This has been taken into account in the statistical analyses.

Food consumption was measured by weighing the pellets given to the animals and weighing the leftovers. This was done twice during the experiment. The first measurement covers the pellet consumption before pairing, over seven days, and the second from the separation of the pairs until the end of the chamber phase, over 15 days. Pellet consumption was not measured during pairing because it was not possible to estimate individual consumption. Body-mass development was monitored by weighing the individuals approximately once per week using an electronic balance (accurate to ± 0.1 g).

For monitoring male maturity and breeding condition (Barkley and Goldman 1977, Fail and Whitsett 1988, Kempenaers et al. 2008) blood samples were collected four times during the experiment for testosterone measurements: (1) right after capture (in laboratory conditions), (2) one week after the manipulations started, (3) during the pairing period, and (4) at the end of the chamber phase. To collect blood we used retro-orbital bleeding. Blood samples were analyzed using the radioimmuno assay method (TESTO-CTK, DiaSorin, Byk-Sangtec Diagonstica, Dietzenbach, Germany), which is based on a competition between unlabeled testosterone and ^{125}I-labeled testosterone for testosterone-specific antibody binding sites. We adapted the test for bank voles by using 15 µL plasma instead of 50 µL, the standard for human testing (see Mills et al. 2007).

The time of vaginal opening as a proxy for maturation in females was observed approximately every five days until the separation of mating pairs (Ergon et al. 2001), after which we started to monitor possible pregnancies. All procedures conducted were approved by the Finnish State Committee for Animal Experimentation (license code: ESLH-2008-05258/Ym-23).

Field enclosure phase

To study possible trade-offs, we tested whether the onset of breeding in predictable vs. unpredictable spring conditions has a long-term effect on subsequent breeding success and survival. For that, we continued the experiment with an enclosure phase in June–July 2011. Following the chamber phase, all the voles except females already with pups were released into eight large outdoor enclosures, each 0.25 ha in size. Voles were distributed over the enclosures according to their original treatment group in the chambers, so only individuals from the same treatment were put together. There were two enclosures per treatment group (ST, HF; ST, LF; VT, HF; VT, LF) and 8–10 voles per enclosure (approximately five males and four females per enclosure). Different treatment groups were distributed randomly across the eight enclosures. Voles were kept in the enclosures for 15 days, which is enough time for two behavioral estruses to occur (Koskela et al. 1996) and to achieve differences in timing and rate of reproduction. After 15 days, the voles were recaptured, females were weighed and taken to the laboratory, and males were released back into the wild at their original capture site. Females were kept at 22° $\pm$ 1°C and fed with standard mice pellets (Labfor R36, Lantmännen, Kimstad, Sweden). Date of parturition and litter size was monitored. Litters were weighed right after birth, and at ages of four, 10, and 20 days. At weaning age (20 days), every pup was sexed and weighed individually. Weather during the field enclosure phase was typical for early summer in central Finland, with average 16°C daily temperature and precipitation of 80 mm/month (Finnish Meteorological Institute).

Statistical analyses.—Statistical analysis was performed using SAS v. 9.1 (SAS Institute, Cary, North Carolina, USA) and SPSS v. 18 (IBM, Armonk, New York, USA). Data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Means are presented with their standard errors (SE), and $P < 0.05$ was the threshold for significance. Daily food consumption was calculated by dividing the amount of food eaten by the number of days between measurements. Food consumption, body mass, and testosterone-level development (for all males that were paired) were analyzed using repeated mixed models with time as the repeated fixed factor and temperature regime (stable vs. variable) as well as food regime (high vs. low quality) as fixed factors. Sex was also entered as a fixed factor for analyses of food consumption and mass development. Female maturation was calculated using a general linear model (GLM). Day of vaginal opening was entered as a dependent variable and temperature and food regime as fixed factors. To test for potential delayed effects of temperature or food regime during the field enclosure phase, we used generalized linear mixed models (GLMM's) with either binomial error structure (survival), Poisson error structure (mating day), or Gaussian error structure (litter size and litter masses). Enclosure was included as a random factor to control for nonindependence of data for individuals sharing the same enclosure. For the analysis of litter masses, we also included mother identity as a random factor using the residual option in the SAS glimmix
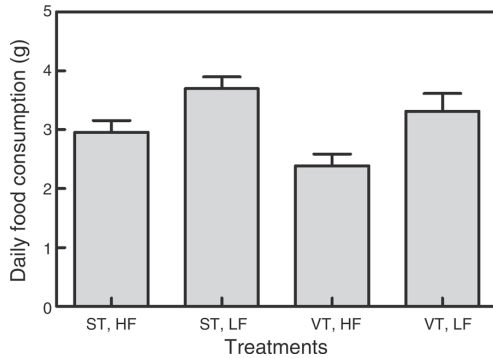
Fig. 2. Daily pellet consumption (least-square [LS] mean with SE) in different treatments; treatments are a combination of stable temperature (ST), variable temperature (VT), high-quality food (HF), and low-quality food (LF). Pellet consumption was significantly affected by the food regime ($F_{1,70} = 12.07$, $P < 0.01$), but not by the temperature treatments ($F_{1,70} = 3.59$, $P = 0.06$).

procedure to account for repeated measures of each litter.

Results

Climate chamber phase

Food consumption.—The food regime significantly affected pellet consumption ($F_{1,70} = 12.07$, $P < 0.01$), which was significantly higher in low-quality food treatments (3.5 ± 0.2 g/d) than in high-quality food treatments (2.7 ± 0.2 g/d, Fig. 2). The trend was similar with both sexes. Individuals experiencing variable temperatures tended to consume less (2.9 ± 0.2 g/d) than individuals experiencing stable temperatures (3.3 ± 0.2 g/d), but this difference was not significant ($F_{1,70} = 3.59$, $P = 0.06$). The interaction of food and temperature
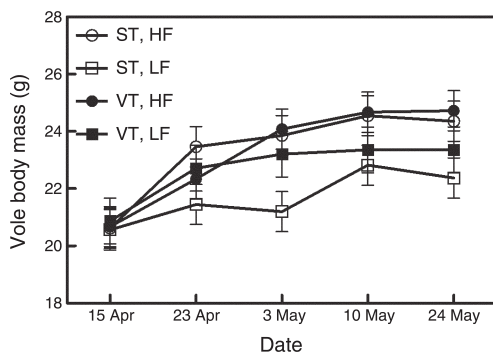

Fig. 3. Body masses (LS mean $\pm$ SE) of voles in different treatments (combinations as are in Fig. 2) during the chamber phase. Body masses were significantly affected by the food regime ($F_{1,72} = 5.36$, $P = 0.02$), but not by the temperature treatments ($F_{1,72} = 0.71$, $P = 0.40$).
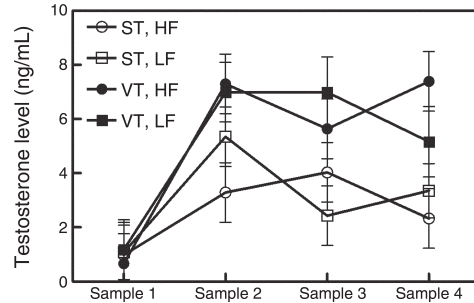

Fig. 4. Testosterone levels (LS mean $\pm$ SE) of the males in different treatments (see Fig. 2) during different phases of the experiment: sample 1, capture day; sample 2, one week after manipulations started; sample 3, while paired; sample 4, last day of the chamber phase. Male testosterone levels were significantly affected by temperature regime ($F_{1,38} = 20.66$, $P < 0.01$), but not by food regime ($F_{1,38} = 0.07$, $P = 0.79$).

regime was not significant ($F_{1,70} = 0.17$, $P = 0.68$). Females consumed on average 2.7 ± 0.2 g/d and males 3.5 ± 0.2 g/d ($F_{1,70} = 10.90$, $P < 0.01$). Pellet consumption increased towards the end of the experiment ($F_{1,70} = 9.02$, $P < 0.01$).

Body mass.—Individuals fed with high-quality food were significantly heavier at the end of the chamber phase (24.0 ± 0.5 g) than individuals fed with low-quality food (22.5 ± 0.5 g; $F_{1,72} = 5.36$, $P = 0.02$, Fig. 3). Temperature regime had no significant effect on body mass (stable vs. variable: 23.0 ± 0.5 g vs. 23.5 ± 0.5 g; $F_{1,72} = 0.71$, $P = 0.40$). The interaction of food and temperature regime was not significant ($F_{1,72} = 1.16$, $P = 0.28$). Males were on average 10% heavier than females throughout the chamber phase ($F_{1,72} = 36.73$, $P < 0.01$), but body-mass development was comparable between the sexes. Body mass increased steadily over time in all treatments ($F_{1,72} = 9.05$, $P < 0.01$, Fig. 3).

Initiation of breeding.—Male testosterone levels (samples 2–4) were clearly affected by temperature regime ($F_{1,38} = 20.66$, $P < 0.01$, Fig. 4). Males from variable temperature treatments had on average 45% higher testosterone levels than males from stable temperature treatments. Food, on the other hand, did not affect testosterone levels ($F_{1,38} = 0.07$, $P = 0.79$), nor did the interaction between food and temperature ($F_{1,38} = 0.49$, $P = 0.50$). Testosterone levels did not significantly change over time, however, a clear increase was observed from the time of capture (sample 1) until the second sampling, which occurred one week after the treatments started (Fig. 4). Male testosterone levels appeared to be independent of body mass ($r = 0.132$, $P = 0.182$).

At the end of the experiment, all but two females were mature. Temperature treatment also affected female maturation, but in an opposite manner than in males. Females experiencing stable temperatures matured on average 6 d earlier than females experiencing variable
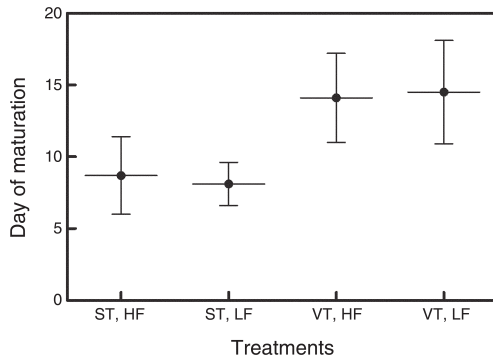
FIG. 5. Day of maturation in females (mean ± SE) in different treatments (see Fig. 2) during the chamber phase. Female maturation was affected by temperature regime ($F_{1,30}$ = 4.43, P = 0.04), but not by food ($F_{1,30}$ < 0.01, P = 0.96).

temperatures (GLM, $F_{1,30}$ = 4.43, P = 0.04, Fig. 5). As with males, food did not seem to affect female maturation ($F_{1,30}$ < 0.01, P = 0.96), nor did their interaction ($F_{1,30}$ = 0.03, P = 0.87).

Because of the difficulties of breeding wild voles in climate chambers, we were not able to get sufficient results on the actual onset of breeding in the chamber phase. Only three females gave birth, one from treatment VT, LF and two from treatment ST, LF.

Field enclosure phase

Survival.—Seventy-three percent of voles (52 out of 71) survived the field enclosure phase. Temperature regime experienced in the laboratory had a significant long-term effect on survival ($F_{1,55}$ = 6.62, P = 0.01). Individuals from stable temperatures had a higher survival probability (0.86 ± 0.06) than individuals from variable temperatures (0.57 ± 0.09). Food and the interaction of food and temperature did not affect survival ($F_{1,55}$ = 0.49, P = 0.49, and $F_{1,55}$ = 6.08, P = 0.30 respectively). Males and females survived similarly well ($F_{1,55}$ = 0.26, P = 0.61).

Onset of breeding and litter size.—In total, 21 females gave birth. From the parturition date we calculated the number of days spent in the enclosures before mating, based on a pregnancy length of 18 days (Buchhalczyk 1970). There was no significant long-term effect of temperature (stable vs. variable; 6.4 ± 0.8 vs. 8.2 ± 0.9, $F_{1,8}$ = 2.11, P = 0.18) or food (high vs. low quality; 7.5 ± 0.8 vs. 7.0 ± 0.9, $F_{1,8}$ = 0.12, P = 0.74, interaction not significant) on mating day. Litter sizes were similar in all treatments (temperature, $F_{1,6}$ = 0.83, P = 0.40; food, $F_{1,6}$ = 0.34, P = 0.58; interaction not significant). Body mass of the mother measured before release into the enclosures did not affect litter size ($F_{1,8}$ = 3.55, P = 0.11). Litter masses were significantly affected by mother's body mass at release to the enclosures ($F_{1,27}$

= 4.41, P = 0.04), but not by the temperature or food regime experienced by the mother in the chamber phase (temperature, $F_{1,27}$ = 0.03, P = 0.86; food, $F_{1,27}$ = 0.10, P = 0.75; interaction not significant).

DISCUSSION

Our results show clear sex differences in the response to environmental cues regulating the onset of reproduction in bank voles. As hypothesized, females preferred stable temperature conditions over variable temperature conditions, with the result that female maturation occurred significantly earlier in the stable temperature treatment than the variable temperature treatment. However, our assumption predicting males to be less sensitive to the temperature regime proved inaccurate, as male maturation, measured as an increase in testosterone level (Wingfield et al. 1990), occurred earlier and was more pronounced under variable temperatures. While food quality had a strong effect on vole body mass in both sexes, there was no evidence for an effect on the onset of breeding. As an indicator of a possible reproductive trade-off, our results show that unstable climatic conditions experienced during maturation in early spring can have a negative delayed effect on survival later in the breeding season.

With our temperature manipulations, we generated simplified simulations representing unstable conditions characteristic of early spring and the more stable and predictable climatic conditions normally occurring later in spring and early in summer. Our results suggest clear sex-specific responses to environmental cues in bank voles. In earlier studies conducted by Daketse and Martinet (1977) and Spears and Clarke (1987), field voles (*Microtus agrestis*) were exposed to different day lengths, temperatures, and diets. Both studies reported enhanced gonadal growth in males exposed to long days and cool temperatures (4°C and 5°C respectively) compared to warmer conditions: 18°C, in Spears and Clarke (1987), 22°C and 33°C in Daketse and Martinet (1977). However, absolute temperatures differed between treatments in these experiments, while in the present study mean temperatures were the same among treatments. There are also conflicting findings with mice and hamsters, which have been noticed to suppress their reproductive functions under cold temperatures (Hoffman et al. 1965, Pryor and Bronson 1981). Thus, without more precise data, one can only speculate on the ultimate cue behind the advanced maturation in males in the variable temperature treatment. Perhaps for northern rodents, like bank voles, which overwinter under the insulating snow cover with rather stable temperatures (Marchand 1996), the strongly fluctuating temperatures combined with accurate above-snow light rhythm indicate snowmelt and the onset of spring. Bank vole males, who typically attain their physiological breeding condition earlier than females (Kaikusalo 1972), may have used this as a cue for the oncoming breeding season. Females, on the other hand, who carry

PLATE 1. Bank vole (*Myodes glareolus*) is a seasonally breeding, small boreal rodent. The breeding season of the species usually occurs from April to September. Photo credit: M. Haapakoski.

the majority of reproductive costs, may use a certain stableness of temperature together with day length as a cue to induce breeding.

Together with climatic conditions, food is generally considered to be one of the main environmental cues affecting the onset of breeding in seasonal environments (Bronson 1989), and in many species, food abundance and quality is used to fine-tune the optimal timing for reproduction in accordance with day length (e.g., Vaughan and Keith 1981, Karasov and Martínez del Rio 2007). In female rodents, sufficient dietary protein is important for maturation and reproductive success (McAdam and Millar 1999, Von Blanckenhagen et al. 2007). In addition, pup growth can be affected by maternal dietary protein level (reviewed by Speakman 2008). For males, on the other hand, a high-protein diet has been shown to act as a competitive advantage, as female meadow voles consider scent marks of males on a high-protein diet most attractive (Ferkin et al. 1997, Hobbs and Ferkin 2011). Consequently, it was surprising that food quality had no detectable effect on maturation and reproduction in either female or male bank voles. Food consumption and body-mass development followed a similar pattern in both sexes. Individuals with a high-protein diet ate less but were heavier than those with a low-protein diet, most likely due to the higher nutritional value of the food. Animals can, to some extent, compensate for low-quality food with quantity, and by growing larger intestines to utilize poor-quality food more efficiently (Gross et al. 1985). Thus, our low-protein diet may have been too good for distinct differences in reproduction to appear between treatments. In an earlier study, field voles were provided

with pellets containing 4%, 8%, 16%, or 24% protein, and only individuals with the 4% diet showed suppression in their reproductive condition (Spears and Clarke 1987). Hence, our results suggest that breeding in bank voles is not as sensitive to protein availability as expected, and food quality is not used as the main cue for optimizing the onset of breeding. However, manipulating the amount of food could generate stronger responses, as several studies have shown that food restriction suppresses reproductive functions in rodents (Bronson 1989).

Irrespective of the mechanism, our results support the general idea that different reproductive costs between the sexes lead to different responses to environmental cues (reviewed by Ball and Ketterson 2008). Due to the misbalanced reproductive investment per gamete in many mammal and bird species, male reproductive fitness is said to be more affected by sexual selection, whereas fecundity selection should have a stronger impact on female fitness. Thus, a substantial part of the unequal costs of reproduction results from the divergent trade-offs males and females face (reviewed by Ball and Ketterson 2008). For example, early onset of breeding under risky environmental conditions extends the breeding season and increases the competitive value and fitness of the individual, in the case of successful reproduction. However, if the breeding attempt fails, it is usually more costly for females. In deer mice (*Peromyscus maniculatus*), early-breeding females showed a higher variance in breeding success, and they also survived more poorly than late breeders (Fairbairn 1977). However, early onset of breeding caused no higher mortality in males (Fairbairn 1977).

To test for possible reproductive trade-offs caused by the reproductive decisions made in early spring, our experiment was continued in large outdoor enclosures, where reproduction and survival were monitored. No significant delayed effects on reproduction were found, but early summer survival was affected by the experienced climatic conditions in spring. Both females and males from the stable temperature treatments survived better than individuals from variable temperature treatments, while food again had no effect. Thus, our results suggest that in bank voles, there is a trade-off between early reproduction (here, maturation) under unstable conditions and survival. Unlike in deer mice (Fairbairn 1977), in bank voles, this was the same for both sexes. However, for male bank voles, early maturation should nevertheless provide a competitive advantage. As population density increases with the season (Henttonen 2000), so does the intensity of male–male competition. Thus, males (who provide no parental care) can afford the risk of dying earlier and should invest strongly on the fertilization of eggs early in the breeding season. Here, the differences in survival in males might be explained by the higher testosterone levels in individuals from variable temperature treatments. Maintaining high testosterone levels is energy-consuming and testosterone causes immunosuppression (Mills et al. 2009). Additionally, males with high testosterone levels move more under natural conditions, which exposes them to higher predation risk (Mills et al. 2009). Male behavior may have also affected females, because mating attempts of males can put both males and females at a risk of being depredated. Though male bank voles cannot force females to copulate, male avoidance and resistance is also energy consuming for females.

The optimal onset of reproduction in a seasonal environment is regulated by environmental cues. It has been shown that due to different selection pressures, females and males may use different cues, or respond differently to the same cues. This phenomenon has gained relatively little consideration, and the physiological mechanisms behind these differences are not well known (Ball and Ketterson 2008). As sexual reproduction requires both sexes, natural selection should always synchronize the onset of breeding independent of the cues used by males or females. However, in the case of rapid environmental change, as is occurring globally, the divergent cues may lead to a reproductive mismatch between the sexes, and also impair the reliability of the cues indicating environmental suitability for reproduction. This could notably decrease the fitness of many seasonally breeding species.

Literature Cited

Ankney, C., and C. MacInnes. 1978. Nutrient reserves and reproductive performance of female Lesser Snow Geese. Auk 95:459–471.

Ball, G. F., and E. D. Ketterson. 2008. Sex differences in the response to environmental cues regulating seasonal reproduction in birds. Philosophical Transactions of the Royal Society B 363:231–246.

Barkley, M. S. M., and B. D. B. Goldman. 1977. A quantitative study of serum testosterone, sex accessory organ growth, and the development of intermale aggression in the mouse. Hormones and Behavior 8:208–218.

Bergeron, J., and L. Jodoin. 1987. Defining "high quality" food resources of herbivores: the case for meadow voles (*Microtus pennsylvanicus*). Oecologia 71:510–517.

Bêty, J., G. Gauthier, and J. Giroux. 2003. Body condition, migration, and timing of reproduction in Snow Geese: a test of the condition-dependent model of optimal clutch size. American Naturalist 162:110–121.

Bondrup-Nielsen, S., and R. A. Ims. 1986. Reproduction and spacing behaviour of females in a peak density population of *Clethrionomys glareolus*. Holarctic Ecology 9:109–112.

Bronson, F. H. 1989. Mammalian reproductive biology. University of Chicago Press, Chicago, Illinois, USA.

Bronson, F. H. 2009. Climate change and seasonal reproduction in mammals. Philosophical Transactions of the Royal Society B 364:3331–3340.

Buchhalczyk, A. 1970. Reproduction, mortality and longevity of the bank vole under laboratory conditions. Acta Theriologica 15:153–176.

Bucyanayandi, J., and J. Bergeron. 1990. Effects of food quality on feeding patterns of meadow voles (*Microtus pennsylvanicus*) along a community gradient. Journal of Mammalogy 390–396.

Bujalska, G. 1990. Social system of the bank vole, *Clethrionomys glareolus*. Pages 155–167 *in* R. H. Tamarin, R. S. Ostfeld, S. R. Pugh, and G. Bujalska, editors. Social systems and population cycles in voles. Springer, Basel, Switzerland.

Butet, A., and Y. R. Delettre. 2011. Diet differentiation between European arvicoline and murine rodents. Acta Theriologica 56:297–304.

Daan, S., C. Dijkstra, R. Drent, and T. Meijer. 1988. Food supply and the annual timing of avian reproduction. Proceedings of the International Ornithological Congress 19:392–407.

Daketse, M., and L. Martinet. 1977. Effect of temperature on the growth and fertility of the field-vole, *Microtus arvalis*, raised in different daylength and feeding conditions. Annales de Biologie Animale Biochimie Biophysique 17:713–721.

Drent, R. H., and S. Daan. 1980. The prudent parent: energetic adjustments in avian breeding. Ardea 68:225–252.

Dunn, P. 2004. Breeding dates and reproductive performance. Advances in Ecological Research 35:69–87.

Eccard J. A., and H. Ylönen. (2006. Adaptive food choice of bank voles in a novel environment: choices enhance reproductive status in winter and spring. Annales Zoologici Fennici 43:2–8.

Ergon, T., X. Lambin, and N. C. Stenseth. 2001. Life-history traits of voles in a fluctuating population respond to the immediate environment. Nature 411:1043.

Fail, P. A. P., and J. M. J. Whitsett. 1988. Influence of photoperiod, ambient temperature and melatonin on testosterone synthesis and release during reproductive maturation in male deer mice. Journal of Andrology 9:21–30.

Fairbairn, D. J. 1977. Why breed early? A study of reproductive tactics in *Peromyscus*. Canadian Journal of Zoology 55:862–871.

Ferkin, M. H., E. S. Sorokin, R. E. Johnston, and C. J. Lee. 1997. Attractiveness of scents varies with protein content of the diet in meadow voles. Animal Behaviour 53:133–141.

Futuyma, D. J. 2005. Evolution. Sinauer, Sunderland, Massachusetts, USA.

Gebczynska, Z. 1983. Feeding habits. Acta Theriologica 28:40–49.

Gross, J., Z. Wang, and B. Wunder. 1985. Effects of food quality and energy needs: changes in gut morphology and capacity of *Microtus ochrogaster*. Journal of Mammalogy 66:661–667.

Gwinner, E. 1986. Circannual rhythms: endogenous annual clocks in the organization of seasonal processes. Springer-Verlag, Berlin, Germany.

Hahn, T. P., T. Boswell J. C. Wingfield, and G. F. Ball. 1997. Temporal flexibility in avian reproduction. Pages 39–80 *in* V. Nolan, E. D. Ketterson, and C. Thompson, editors. Current ornithology. Volume 14. Springer, Basel, Switzerland.

Henttonen, H. 2000. Long-term dynamics of the bank vole *Clethrionomys glareolus* at Pallasjaervi, northern Finnish taiga. Polish Journal of Ecology 48:87–96.

Hobbs, N. J., and M. H. Ferkin. 2011. Dietary protein content affects the response of meadow voles, *Microtus pennsylvanicus*, to over-marks. Acta Ethologica 14:57–64.

Hoffman, R. A., R. J. Hester, and C. Towns. 1965. Effect of light and temperature on the endocrine system of the golden hamster (*Mesocricetus auratus* Waterhouse). Comparative Biochemistry and Physiology 15:525–533.

Jones, L. R. 1986. The effect of photoperiod and temperature on testicular growth in captive black-billed magpies. Condor 88:91–93.

Kaikusalo, A. 1972. Population turnover and wintering of the bank vole, *Clethrionomys glareolus* (Schreb.), in southern and central Finland. Annales Zoologici Fennici 9:219–224.

Karasov, W. H., and C. Martinez del Rio. 2007. Physiological ecology: how animals process energy, nutrients, and toxins. Princeton University Press, Princeton, New Jersey, USA.

Kempenaers, B., A. Peters, and K. Foerster. 2008. Sources of individual variation in plasma testosterone levels. Philosophical transactions of the Royal Society of London B 363:1711–1723.

Koivula, M., E. Koskela T. Mappes, and T. Oksanen. 2003. Cost of reproduction in the wild: manipulation of reproductive effort in the bank vole. Ecology 84:398–405.

Kokko, H. 1999. Competition for early arrival in migratory birds. Journal of Animal Ecology 68:940–950.

Koskela, E., T. J. Horne, T. Mappes, and H. Yloenen. 1996. Does risk of small mustelid predation affect the oestrous cycle in the bank vole, *Clethrionomys glareolus*? Animal Behaviour 51:1159–1163.

Lindroth, R. L., and G. Batzli. 1984. Food habits of the meadow vole (*Microtus pennsylvanicus*) in bluegrass and prairie habitats. Journal of Mammalogy 65:600–606.

Marchand, P. J. 1996. Life in the cold: an introduction to winter ecology. University Press of New England, Lebanon, New Hampshire, USA.

McAdam, A., and J. Millar. 1999. Dietary protein constraint on age at maturity: an experimental test with wild deer mice. Journal of Animal Ecology 68:733–740.

Meijer, T., U. Nienaber, U. Langer, and F. Trillmich. 1999. Temperature and timing of egg-laying of European Starlings. Condor 101:124–132.

Millar, J. S., and L. W. Gyug. 1981. Initiation of breeding by northern *Peromyscus* in relation to temperature. Canadian Journal of Zoology 59:1094–1098.

Mills, S. C., A. A. Grapputo, I. Jokinen, E. Koskela, T. Mappes, T. A. Oksanen, and T. Poikonen. 2009. Testosterone-mediated effects on fitness-related phenotypic traits and fitness. American Naturalist 173:475–487.

Mills, S. C. S., A. A. Grapputo, E. E. Koskela, and T. T. Mappes. 2007. Quantitative measure of sexual selection with respect to the operational sex ratio: a comparison of selection indices. Proceedings of the Royal Society B 274:143–150.

Nilsson, J. 1989. Causes and consequences of natal dispersal in the Marsh Tit, *Parus palustris*. Journal of Animal Ecology 58:619–636.

Norris, K. 1993. Seasonal variation in the reproductive success of Blue Tits: an experimental study. Journal of Animal Ecology 62:287–294.

Perrins, C. M. 1970. The timing of birds' breeding season. Ibis 112:242–255.

Pryor, S., and F. H. Bronson. 1981. Relative and combined effects of low temperature, poor diet, and short daylength on the productivity of wild house mice. Biology of Reproduction 25:734–743.

Robbins, C. T. 1993. Wildlife feeding and nutrition. Academic Press, Waltham, Massachusetts, USA.

Sharpe, S., and J. S. Millar. 1991. Influence on the variation in initiation of breeding in *Peromyscus maniculatus*. Canadian Journal of Zoology 69:698–705.

Skinner, W. R., R. L. Jefferies, T. J. Carleton, R. F. Rockwell, and K. F. Abraham. 1998. Prediction of reproductive success and failure in Lesser Snow Geese based on early season climatic variables. Global Change Biology 4:3–16.

Speakman, J. R. 2008. The physiological costs of reproduction in small mammals. Philosophical Transactions of the Royal Society of London B 363:375–398.

Spears, N., and J. R. Clarke. 1987. Effect of nutrition, temperature and photoperiod on the rate of sexual maturation of the field vole (*Microtus agrestis*). Journal of Reproduction and Fertility 80:175–181.

Trebaticka, L., T. Ketola, I. Klemme, J. A. Eccard, and H. Ylönen. 2007. Is reproduction really costly? Energy metabolism of bank vole (*Clethrionomys glareolus*) females through the reproductive cycle. Ecoscience 14:306–313.

Trivers, R. L. 1972. Parental investment and sexual selection. Pages 136–207 *in* B. Campbell, editor. Sexual selection and the descent of man, 1871–1971. Aldine Publishing, Chicago, Illinois, USA.

Van Noordwijk, A., R. H. McCleery, and C. M. Perrins. 1995. Selection for the timing of Great Tit breeding in relation to caterpillar growth and temperature. Journal of Animal Ecology 64:451–458.

Vaughan, M. R., and L. B. Keith. 1981. Demographic response of experimental snowshoe hare populations to overwinter food shortage. Journal of Wildlife Management 45:354–380.

Visser, M. E., L. J. Holleman, and S. P. Caro. 2009. Temperature has a causal effect on avian timing of reproduction. Proceedings of the Royal Society of London B 276:2323–2331.

Von Blanckenhagen, F., J. A. Eccard, and H. Ylönen. 2007. Animal protein as a reproductive constraint in spring reproduction of the bank vole. Ecoscience 14:323–329.

Wingfield, J. C., R. E. Hegner, A. M. Dufty, and G. F. Ball. 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. American Naturalist 136:829–846.

IV

VALIDATION OF ENZYME IMMUNOASSAYS AND EVALUATION OF FACTORS AFFECTING THE LEVELS OF FECAL STEROID METABOLITES IN BANK VOLES (MYODES GLAREOLUS)

by

Saana Sipari, Rupert Palme & Hannu Ylönen 2014

Manuscript

VALIDATION OF ENZYME IMMUNOASSAYS AND EVALUATION OF FACTORS AFFECTING THE LEVELS OF FECAL STEROID METABOLITES IN BANK VOLES (*MYODES GLAREOLUS*)

Saana Sipari[1], Rupert Palme[2] and Hannu Ylönen[1]

1 Department of Biological and Environmental Science, Konnevesi Research Station, University of Jyväskylä, P.O.Box 35, FI-40014 Jyväskylä, Finland; 2 Institute of Physiology, Pathophysiology and Biophysics, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

ABSTRACT

Using fecal samples for endocrinological studies has many advantages. The sampling procedure is noninvasive and it enables frequent sampling without compromising the well-being of the animal. However, for a meaningful use of fecal samples, the species specific circadian fluctuation of hormones, gut passage time, excretion route and sex specific differences should always be taken into account. Here, we tested the applicability of two enzyme immunoassays (EIA) for assessing fecal corticosterone metabolite and testosterone metabolite levels in bank voles (Myodes glareolus) and evaluated the factors affecting the levels of these steroids. To test the suitability of the 5α-pregnane-3β,11β,21-triol-20-one EIA for measuring fecal corticosterone metabolites we performed an adrenocorticotropic hormone (ACTH) challenge test. To study the metabolism and excretion of corticosterone we also performed a radiometabolism study. For measuring testosterone metabolites we used a testosterone EIA, measuring 17ß-hydroxyandrogens. To evaluate the sensitivity of the testosterone EIA we used females as a reference group, as females are known to have significantly lower testosterone levels than males. A radiometabolism study was also performed with testosterone. Both of the EIAs (5α-pregnane-3β,11β,21-triol-20-one EIA and the17ß-hydroxyandrogen EIA) proved to be suitable for measuring the given fecal steroid metabolites in bank voles. Males had significantly higher corticosterone metabolite levels than females and their main excretion route was via feces, whereas in females the ratio between feces and urine was nearly half-and-half. The time course of excretion was similar in both sexes. As expected, males had significantly higher testosterone metabolite levels than females. The main excretion route was via feces in both sexes. Time of the day had a significant effect on the excretion of both steroids. The bank vole is a commonly used model species in behavioral and ecophysiological studies. Thus, presenting a validated method for noninvasive monitoring of corticosterone and testosterone secretion is of high relevance.

Key words: Non-invasive, feces, corticosterone, testosterone, ACTH challenge, radiometabolism, bank vole, *Myodes glareolus*

INTRODUCTION

The endocrine system is responsible for numerous essential bodily functions in animals, including development from a fetus to an adult, as well as everyday maintenance of physiological homeostasis (Campbell and Reece 2005). Hormones also play a major role in the behavior of animals and thus provide a powerful tool for research of animal behavior and physiology. However, endocrinological studies can be problematic, as they often require blood sampling or another invasive procedure. Thus, the sampling procedure itself can significantly alter the results, leading to incorrect conclusions (Gartner *et al.* 1980, Haemisch *et al.* 1999). Also, some of the traditional sampling methods are considered unethical. In order to avoid these problems, researchers have shown increasing interest in noninvasive methods for hormonal sampling. Saliva, hairs, urine and feces have been under research in order to obtain a reliable, but noninvasive method for studying hormonal status (Palme *et al.* 2005, Keckeis *et al.* 2012, Behringer *et al.* 2013, Zeugswetter *et al.* 2013). With many species fecal samples are shown to be a practical method for this purpose (Touma and Palme 2005, Sheriff *et al.* 2011).

Besides practical and ethical issues, using fecal samples for endocrinological studies have also other advantages compared to blood sampling. Hormone concentration measured from blood samples provides only a snapshot of the hormonal status of the animal, which is problematic particularly in the case of hormones with strong diel fluctuation and fast reaction to stimuli, like glucocorticoids. Fecal samples, on the other hand, reflect the hormone secretion for a longer period of time and thus dampen minor and short-term fluctuations in hormone secretion (Palme 2005, Palme *et al.* 2005). However, using samples other than blood for determining the concentration of a given hormone in an animal is not straightforward. The metabolic rate of hormones in a certain species determines the time gap between hormone secretion and the excretion of the metabolites in feces. Thus, in order to correctly interpret results based on fecal analyses one should always know the average gut passage time of the given product (Palme *et al.* 2005). Also sex, age, time of the day and even diet may affect the metabolism (Touma *et al.* 2003, Dantzer *et al.* 2011a). Another important matter is the excretion route of the hormone or its metabolites, which can vary significantly between species (Bahr *et al.* 2000, Bamberg *et al.* 2001, Touma *et al.* 2003, Montiglio *et al.* 2012). For some species the main excretion route is feces while some other may excrete the majority of particular hormone metabolites via urine (Palme et al. 2005). Also, within a given species there can be significant sex differences in excretion routes (Touma *et al.* 2003).

There can be some hormone and species specific differences in the ratio of hormone metabolites and actual hormone present in the feces (Palme *et al.* 2005). However, in the case of many groups of hormones, like glucocorticoids, it is safe to say that hormone metabolites are the only measurable content available in the excreta (Palme and Möstl 1997, Touma *et al.* 2003). Thus, immunoassays designed for blood samples are often unsuited for analyzing

fecal samples, as they are targeted for actual hormone molecules rather than their metabolites. Using immunoassays especially designed for hormone metabolites is advisable in order to get reliable results (Möstl et al. 2005). However, hormone metabolites are a highly diverse group of molecules, as the types of metabolites can vary between species and even sexes (Palme *et al.* 2005). Hence, finding a suitable group specific enzyme immunoassay for different species and sexes is of great importance.

Here, we performed a multiphase validation experiment in order to test the applicability of two enzyme immunoassays (EIA) for measuring fecal corticosterone metabolites and testosterone metabolites in bank voles (*Myodes glareolus*). For measuring corticosterone metabolites (CM) we used a 5α-pregnane-3β,11β,21-triol-20-one EIA, which measures metabolites with 5α-3β,11β,-diol structure. It has been shown to be suitable for mice (*Mus musculus* f. *domesticus*) (Touma *et al.* 2003, Touma *et al.* 2004), rabbits (*Oryctolagus cuniculus*) (Monclús *et al.* 2006), rats (*Rattus norvegicus*) (Lepschy *et al.* 2007), hamsters (*Mesocricetus auratus*) (Chelini *et al.* 2010) and several squirrel species (Bosson *et al.* 2009, Dantzer *et al.* 2010, Bosson *et al.* 2013) For testosterone metabolites (TM) we used a testosterone EIA, measuring 17ß-hydroxyandrogens, first described by Palme & Möstl (1994). This particular EIA has been validated also for assessing androgen metabolite concentrations in female and male red squirrels (*Tamiasciurus hudsonicus*) (Dantzer *et al.* 2011a, Dantzer *et al.* 2011b). These particular steroids were chosen for their significant role in behavioral and physiological functions in animals. Stress reactions have an important role in the survival of all animals but in case of prolonged stress it can also strongly affect the reproduction and immune functions of the individual. Corticosterone acts as the main stress hormone especially in many small mammals (Boonstra 2004). Testosterone, on the other hand, is the main reproductive hormone of males in most vertebrate species, including the bank vole. Testosterone levels often indicate the reproductive status (immature vs. mature), but it can also strongly affect the behavior of the individual, making it a very interesting variable for physiological, ethological and ecological studies (Campbell and Reece 2005).

Bank voles are widely used for laboratory experiments as well as field studies among mammal research. Monitoring the endocrine functions in small rodents is often performed using blood samples, collected from the tail vein or from the orbital sinus (Bradshaw 2003). Few studies have been exploiting fecal samples for glucocorticoid measurements in bank voles, but to our knowledge, a proper analytical and biological validation of a method to measure fecal steroid metabolites (CM or TM) is still lacking. Harper & Austad (2000) performed a validation experiment for measuring fecal corticosteroids in red-backed voles (*Myodes gapperi*) with a radioimmunoassay (RIA) for corticosterone, which has been used as a reference for experiments using fecal samples for monitoring stress in bank voles (Ylönen *et al.* 2006, Eccard *et al.* 2011). However, the RIA used in Harper & Austad (2000) was designed to detect the actual hormone rather than its metabolites. Its ability to measure fecal

CM is based on some level of cross-reaction with the metabolites of the parent hormone (Harper and Austad 2000). Thus, a validation of a method designed to measure particularly fecal corticosterone metabolites is needed. The experimental design of our study was mainly based on the design and procedures described by Touma et al. (2003) and Touma et al. (2004), in which the authors validated the suitability of the EIA (for 5α-pregnane-3β,11β,21-triol-20-one) for fecal CM measurements in mice and tested the effects of sex and time of day on metabolism and excretion route of the metabolites using radiometabolism experiment. Additionally, we applied the same principles for testosterone metabolites as well. As the bank vole is a commonly used model species in behavioral and ecophysiological studies this kind of thorough validation study of these particular steroids is of high relevance. To our knowledge, only one radiometabolism experiments with testosterone (Billitti *et al.* 1998) has been described in a small rodent species before this.

MATERIAL AND METHODS

Experimental animals

All 48 animals used in this experiment (24 females and 24 males) were laboratory born (August 2012), adult individuals, descending from a colony of wild captured bank voles from Central-Finland, close to the Konnevesi Research station. Before the experiments, all animals were housed individually in standard mouse cages (43×26×15 cm) with wood shavings and hay as bedding. Light/dark cycle in the laboratory (18L:6D, lights on 6:00 and off 24:00) was a standard long day cycle used in our laboratories during summer. The laboratory was maintained at 22 ± 1˚C. Standard mice pellets (Labfor R36, Lantmännen) and bottled tap water were provided *ad libitum*. Animals were maintained in the laboratory at the Konnevesi Research station in Finland.

Experimental design

The experiments were performed during June - July 2013. Five days before the experiments started all animals were moved to the experimental cages for habituation (Table 1). To facilitate the sampling of feces and urine, and to avoid unnecessary handling of the animals during the experiment, the voles were housed in steel wire cages which enabled the excreta to drop through the bottom of the cage. A plastic container covered with paper towels was placed below each wire cage to collect feces and urine. To provide some shelter for the animals, some hay was added in one corner of the wire cages. The hay was sparse and coarse enough not to hinder the excreta dropping through the bottom of the cage.

Corticosterone

1. *Evaluation of circadian fluctuation*

First, the normal circadian fluctuation of the steroid excretion was observed. Twelve males and 12 females were used for this purpose. Animals received no handling or other physical disturbance before the experiment. Feces sampling was done during 72 hours according to the following time schedule: 09:00, 11:00, 13:00, 15:00, 17:00, 19:00, 21:00, 01:00, 05:00, 09:00, 21:00, second day 09:00, third day 09:00 hours. All fecal samples were collected from the paper covered containers under each cage into plastic Eppendorf-tubes (1.5 ml) using tweezers, and stored in freezer at -20 ^{0}C. Feces clearly contaminated with urine (e.g. feces lying in urine spots) were not collected. In order to not disturb the animals' normal activity rhythms by turning the lights on during night (during the dark phase of their L:D regime) we used head lamps with low light intensity to collect the night samples. No urine was collected for the evaluation of circadian fluctuation.

2. *ACTH challenge*

To stimulate the adrenocortical activity of the voles an ACTH challenge experiment was performed. The same individuals that were used for the circadian fluctuation study were injected with synthetic ACTH (ACTH Depot, Defiante Farmaceutica S.A), each with a dosage of 60 µg/100 g body weight. The ACTH was mixed with sterile isotonic saline solution and the total volume of each injection was 250 µL. The injections were performed at 09:00 am intraperitoneally. This procedure lasted max 4 minutes per animal. Again, feces sampling was performed during the following 72 hours according to the time schedule and procedure described in the circadian fluctuation study. Extraction of fecal steroids for analyzing was conducted according to the method described by Palme & Möstl (1997) and validated for mice by Touma et al. (2003). Briefly, each fecal sample was homogenized and an aliquot of 0.05 g was mixed with 80% methanol and shaken in a multi-vortex. After this, the suspension was centrifuged for 10 min at 2500 x *g.* An aliquot of the supernatant was diluted (1:10) with assay buffer (Tris/HCl 20 mM, pH 7.5) and stored in freezer at -20^{0}C until analysis. To determine the amount of corticosterone metabolites a 5α-pregnane-3β,11β,21-triol-20-one EIA was used. This EIA utilizes a group-specific antibody measuring steroids with 5α-3β,11β-diol structure.

3. *Radiometabolism study: Time course and route of corticosterone metabolite excretion*

To study the metabolism and excretion of corticosterone in bank voles we performed a radio-metabolism study. We selected six males and six females from the original 24 individuals used for the circadian fluctuation study and ACTH challenge. There was 1 week between the end of the ACTH experiment

and the beginning of the metabolism study. These voles were injected intraperitoneally with 740 kBq (20 µCi) of ^{3}H-corticosterone (1,2,6,7-^{3}H-corticosterone, specific activity: 85-105 Ci/mmol (3.145-3.885 TBq/mmol), BIOTREND Chemikalien GmbH, Köln, Germany). The radiolabeled hormone was mixed with sterile isotonic saline solution containing 5% ethanol. The total volume of each injection was 250 µL. Feces sampling was done similar to the circadian fluctuation study and ACTH challenge experiment. To study the excretion route of corticosterone also urine samples were collected. During each sampling the paper towels were renewed and papers with urine spots were collected into small plastic bags and stored at -20 ^{0}C. Before freezing, the urine spots on the paper were circled with a drawing pen to facilitate the analysis procedures later on. To determine the route and time course of the steroid excretion, tested by the radio-metabolism experiments, the analysis procedure followed the methods described in Touma et al. (2003). Briefly, after extraction, the radioactivity of each sample (urine and feces) were measured using a liquid scintillation counter (Tri-Carb 2100TR, Packard Instruments, Meriden, CT USA). Following this, peak excretion samples and thus delay times of urinary and fecal excretion could be calculated. Comparing the ratio of recovered radioactivity in feces and urine indicates the extraction route.

4. *Characterization of fecal corticosterone metabolites and determination of immunoreactivity*

Reversed-phase high performance liquid chromatography (HPLC) was performed to determine the type and relative abundance of fecal ^{3}H-steroid metabolites. Radioactive corticosterone metabolites were separated from four fecal samples (two samples from males and two from females) according to their polarity by using reverse-phase high performance liquid chromatography (HPLC). Radioactivity of the fractions was measured with liquid scintillation counting and immunoreactivity with the 5α-pregnane-3β,11β,21-triol-20-one EIA and for comparison with a corticosterone EIA (Palme and Möstl, 1997). This procedure followed the methods described in Touma et al. (2003).

Testosterone

1. *Evaluation of circadian fluctuation*

The circadian fluctuation study in testosterone followed the same procedure as the one evaluating corticosterone levels (see above), and these two studies were carried out simultaneously in the same laboratory. Twelve males and twelve females were used for this experiment. However, during analysis it was noticed that the samples originally collected for analysing the circadian fluctuation in corticosterone were large enough enabling the analysis with the testosterone EIA as well. Thus, the final sample size for analysing the circadian fluctuation

in testosterone was doubled (24 males and 24 females). We performed no artificial increase of testosterone level, like the ACTH challenge in the corticosterone study. To evaluate the sensitivity of the EIA used for the analyses we used females as a reference group, as females are known to have significantly lower testosterone levels than males.

2. *Radiometabolism study: Time course and route of testosterone metabolite excretion*

To study the time course and route of testosterone excretion, a radio-metabolism study was performed. Six males and six females were selected from the original 24 individuals used for the circadian fluctuation study in testosterone, and were injected intraperitoneally with 740 kBq (20 µCi) of ^{3}H-testosterone (1,2,6,7-^{3}H-testosterone, specific activity: 85-105 Ci/mmol (3.145-3.885 TBq/mmol), BIOTREND Chemikalien GmbH, Köln, Germany). Similar to the radio-metabolism experiment for corticosterone, the radiolabeled testosterone was mixed with sterile isotonic saline solution containing 5% ethanol. The total volume of each injection was 250 µL. Sampling procedures and time schedule were the same as described before. This experiment was carried out simultaneously with the radiometabolism study for corticosterone, using the same facilities.

3. *Characterization of fecal testosterone metabolites and determination of immunoreactivity*

The HPLC separation followed the same procedure as described earlier with corticosterone metabolites, but the immunoreactivity of the fractions was measured with a testosterone EIA and for comparison with an epiandrosterone EIA (Palme and Möstl, 1994).

Statistical analysis

Statistical analyses were performed using R 3.0.3 and IBM SPSS Statistics 20. All data were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests of normality. Steroid metabolite levels in circadian fluctuation studies and ACTH challenge were log-transformed for normality and analysed using linear mixed-effect models fitted by REML. For model selection we used the Akaike information criterion (AIC), model with the lowest AIC value selected for the For circadian fluctuation studies, sex and time were entered as fixed factors, and individual as random factor. When comparing the corticosterone metabolite levels between the circadian fluctuation study (basal level) and ACTH challenge experiment, the model was extended by adding treatment (circadian fluctuation i.e. basal level vs. ACTH) as fixed factor. The route of excretion was analysed by comparing the ratios of recovered radioactivity in urine and fecal samples between males and females by using Welch two sample

t-test for corticosterone metabolites (data normally distributed) and Wilcoxon rank sum test for testosterone metabolites (data not normally distributed). Welch two sample t-test was used for testing the total recovery of the administered radioactivity in males and females. For comparing the excretion rate between females and males and the time course of excretion between urine and feces, we used Pearson correlation for normally distributed data and Spearman's correlation for not normally distributed data.

RESULTS

Corticosterone

1. Evaluation of circadian fluctuation

Time of the day had a significant effect on the excretion of fecal corticosterone metabolites (T_{169} = -3.385, p < 0.001). The highest concentrations in both females and males occurred during afternoon (at 13:00 o'clock) and the lowest levels were observed during mornings (at 9:00 o'clock) and evenings (at 21:00 o'clock; Fig 1). Males had significantly higher corticosterone metabolite levels than females (T_{22} = 6.259, p < 0.001).

2. ACTH challenge

The ACTH administration significantly increased the corticosterone metabolite concentrations in both females and males (T_{333} = -2.465, p = 0.014). A clear peak in the CM levels was observed 6 hours after injection in females and 8 hours after injection in males (Fig 2). The ACTH increased the mean CM concentration over 300 percent compared to the normal circadian variation level at the same time point. Males had significantly higher CM concentrations than females (T_{22} = 3.490, p = 0.002). There were no significant interactions.

3. Time course and route of corticosterone metabolite excretion

There were significant sex-specific differences in the route of corticosterone metabolite excretion. Males excreted on average 69.7 ± 5.1% ^{3}H-corticosterone metabolites via the feces, whereas females excreted on average 51.5 ± 3.9% ($T_{9.353}$ = -2.810, p = 0.02). Consequently, (^{3}H) corticosterone metabolite concentration was significantly higher in the urine of females than in males (males: 30.3 ± 5.1%, females 48.5 ± 3.9%, $T_{9.353}$ = 2.810, p = 0.02; Fig 3). The time course of excretion followed similar trends in males and females (excretion via feces: Pearson correlation r = 0.77, p = 0.003, excretion via urine: Spearman's correlation r = 0.923, p < 0.01). However, there was a clear difference between the time course of excretion between feces and urine (Pearson correlation r = -0.068, p = 0.834). In the feces the peak concentrations were reached around 6 hours after the injection of ^{3}H-corticosterone. However, there seems to be some

variation in the rate of metabolism between individuals, as we observed also another peak in ^{3}H-corticosterone metabolite levels around 16-20 hours after injection (Fig 4a). This pattern was similar in both sexes. In urine the excretion rate was more constant, as the notably highest proportions of radioactivity was recovered 2 hours after the injection (Fig 4b). Only 21% from the originally administered radioactivity was recovered. There were no difference in the degree of the total recovery between sexes ($T_{9.829} = 0.698$, p = 0.501).

4. Characterization of fecal corticosterone metabolites and determination of immunoreactivity

The highest amounts of radioactive metabolites were found in fractions 10 - 50 in males and 10 - 70 in females (Fig 5). There were differences in the metabolites between the sexes. Females produced a wider scale of metabolites than males, some being less polar than the injected corticosterone. The 5α-pregnane-3β,11β,21-triol-20-one EIA detected more metabolites than the corticosterone EIA, which showed only very minor immunoreactivity. The original corticosterone was heavily metabolized.

Testosterone

1. Evaluation of circadian fluctuation

Testosterone metabolite levels were significantly affected by sex ($T_{367} = 12.084$, p < 0.001) and time of the day ($T_{367} = 2.476$, p = 0.014). Males had on average six times higher concentrations than females. There was a significant interaction between sex and time of the day (L-ratio$_6$ = 6.564, p = 0.01). Unlike in CM, the circadian variation in testosterone was less constant (Fig 6). However, the highest concentrations seem to be excreted in the evening and at night.

2. Time course and route of testosterone metabolite excretion

Males excreted more ^{3}H-testosterone metabolites via feces than females but the difference was not significant (males: 83.1 ± 3.7%, females: 70.7 ± 9.0%, W = 12, p = 0.394; Fig 7). The time course of excretion was similar between the sexes (excretion via feces: Spearman's correlation r = 0.559, p = 0.059, excretion via urine: Spearman's correlation r = 0.783, p = 0.003). Testosterone metabolites were excreted more through feces than corticosterone metabolites ($T_{21.18}$ = -2.5023, p = 0.021). The excretion rate in testosterone differed between feces and urine (Spearman's correlation r = 0.378, p = 0.226). Similar to CM, excretion via the urine was faster than via the feces. The highest concentrations of ^{3}H-TM were observed 16 hours after the administration of the hormone, whereas most of the radioactivity in urine was recovered 4 - 6 hours after the injection (Fig 8). From the originally administrated radioactivity 31 % was recovered in males

and only 13 % in females. The difference between sexes was significant ($T_{8.892}$ = - 4.973, p < 0.01).

3. *Characterization of fecal testosterone metabolites and determination of immunoreactivity*

There was one prominent peak of radioactivity found in both sexes, accompanied with several small ones (Fig 9). In females, the main peak eluted between fractions 40-45 and in males between fractions 32 - 36. Unfortunately the testosterone EIA was not able to detect the main metabolite, but it detected many of the less prominent ones. Similar to corticosterone, the original testosterone was heavily metabolized.

DISCUSSION

Our result show that a 5α-pregnane-3β,11β,21-triol-20-one EIA (measuring corticosterone metabolites with a 5α-3β,11β-diol structure), established and first described by Touma et al (2003) can be used for reliable measurements of fecal corticosterone metabolites in bank voles. The EIA was able to detect the ACTH induced increase in CM concentrations in both sexes, as well as many of the formed metabolites in our radiometabolism study. Also, the testosterone EIA (measuring 17ß-hydroxyandrostanes, first described in Palme & Möstl 1994) showed to be suitable for assessing fecal testosterone metabolites in this species.

We observed a strong circadian fluctuation in fecal CM concentrations in bank voles. The highest concentrations in both sexes tended to occur during afternoon hours, but females also expressed high levels in the early morning. Opposite to e.g. mice (Touma *et al.* 2003) the amounts of measured fecal CM were significantly higher in males than in females. A similar dimorphism was observed in Syrian hamsters and common hamsters caused by the larger adrenal glands and greater steroid production in males. (Huhman *et al.* 2003, Franceschini *et al.* 2007, Chelini *et al.* 2010). However, sometimes the chosen EIA detects more CMs formed by the other sex, which can cause sex differences in the results (Touma *et al.* 2003). The variance between individuals was high. The ACTH challenge experiment confirms the sensitivity of the selected EIA, as there was a clear and significant increase in the CM concentrations in both sexes. The peak CM values were detected around 6-8 hours after the administration of the ACTH in the feces. A similar time lag was observed in mice (Touma *et al.* 2003), spiny mice (*Acomys cahirinus*) (Nováková *et al.* 2008) and even in red squirrels (Dantzer *et al.* 2010) whereas in syrian hamster, member of the same Family as bank voles, the gut passage time was 3 - 4 times longer (Chelini *et al.* 2010). This time indicates the average gut passage time of corticosterone metabolites in bank voles, which is a highly important piece of information when examining the endocrine status of animals based on excreta. The radiometabolism studies confirm this time lag. In urine the excretion time of the ^{3}H-CM was 2 hours after the injection. The ACTH challenge as well as the

radiometabolism study showed that, in case of fecal samples, there was variation between individuals in the excretion rate of CM. This could be a consequence of a varying activity rhythm in bank voles. Bank voles are largely nocturnal but they have a polyphasic activity pattern consisting of several activity bouts also during the day (Ylönen 1988, Halle 2000). The occurrence of these activity bouts can vary between individuals which may appear as inconsistency in the results. Nevertheless, adding up the diel variation in fecal CM concentrations and the observed gut passage time it seems that on average the strongest corticosterone production occurs during the early hours at dawn. The radiometabolism studies also revealed that, similar to mice (Touma et al. 2003), there are clear sex-specific differences in the excretion route of CM in bank voles. Males excrete around 70 % of CM via feces and 30 % via urine, whereas in females the ratio is nearly half-and-half (51.5 % vs. 48.5%). This difference in excretion routes between sexes is highly important when comparing the endocrine status of females and males. The high performance liquid chromatography (HPLC) showed that there were also some sex-specific differences in the coricosterone metabolites formed and excreted in feces. Females produced a wider range of metabolites, some with lower polarity than in males. The HPLC also shows that original plasma corticosterone is heavily metabolized, which indicates that there are almost no measurable amounts of corticosterone left in the feces, only metabolites. This strongly supports the use of EIAs particularly designed for detecting metabolites, rather than the actual steroid when using fecal samples (Möstl *et al.* 2005)

For testing the sensitivity of the selected EIA for testosterone metabolites, we performed no stimulation experiment but compared the natural TM concentrations of males and females. The EIA was able to detect even the low concentrations of TM in females, and thus, to show the expected differences in TM concentrations between males and females. There was a circadian fluctuation in TM levels but with no clear pattern. However, the highest TM concentrations in males occurred at night. Based on the radio-metabolism study, it appears that testosterone is slower to metabolize and excrete than corticosterone. The highest values of TM in feces were observed 16 hours after injection, whereas the peak values in urine occurred after 6 hours. These time lags are similar to the results reported with mice and deer mice (*Peromyscus maniculatus*) (Billitti *et al.* 1998). Adding up the gut passage time, the highest testosterone production seems to occur around morning and forenoon. With the excretion route there was no significant difference between the sexes. The main excretion route for TM was via feces (circa 83 % in males and 72 % in females). The HPLC showed one highly prominent peak of radioactivity in both sexes, accompanied with some smaller ones. Unfortunately the testosterone EIA was not able to detect the main metabolite peak. However, the physiological validation showed that the EIA detected the expected difference in the TM concentration between males and females based on those less prominent metabolites formed and excreted. The HPLC also shows that there are no

unwanted cross reactions in our EIA. Similar to corticosterone, the original testosterone was heavily metabolized.

With this validation experiment we were able to provide some essential background knowledge of the endocrine physiology in bank vole, and validate an EIA (5α-pregnane-3β,11β,21-triol-20-one EIA) for measuring fecal corticosterone metabolites and a testosterone EIA for measuring fecal androgen metabolites (17ß-hydroxyandrostanes) in that species.

Using fecal samples for endocrinological studies can be a very valuable tool. It enables frequent and noninvasive sampling with minimal side effects, compared to blood sampling. Also, it can be easily applied in the laboratory or in the field. However, for a reliable and meaningful use of fecal samples, the species specific circadian fluctuation of the particular hormone, gut passage time, excretion route and sex specific differences should always be taken into account. Bank voles are commonly used species both in laboratory and field experiments, and thus, we are confident that presenting a validated method for noninvasive monitoring of corticosterone and testosterone secretion in bank voles will be of great use and may enhance the use of noninvasive methods in endocrinological studies.

REFERENCES

Bahr N., Palme R., Möhle U., Hodges J. & Heistermann M. 2000. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. *Gen. Comp. Endocrinol.* 117: 427-438.

Bamberg E., Palme R. & Meingassner J.G. 2001. Excretion of corticosteroid metabolites in urine and faeces of rats. *Lab. Anim.* 35: 307-314.

Behringer V., Borchers C., Deschner T., Möstl E., Selzer D. & Hohmann G. 2013. Measurements of salivary alpha amylase and salivary cortisol in hominoid primates reveal within-species consistency and between-species differences. *PloS one* 8: e60773.

Billitti J.E., Lasley B.L. & Wilson B.W. 1998. Development and validation of a fecal testosterone biomarker in Mus musculus and Peromyscus maniculatus. *Biol. Reprod.* 59: 1023-1028.

Boonstra R. 2004. Coping with changing northern environments: the role of the stress axis in birds and mammals. *Integr. Comp. Biol.* 44: 95-108.

Bosson C.O., Palme R. & Boonstra R. 2009. Assessment of the stress response in Columbian ground squirrels: laboratory and field validation of an enzyme immunoassay for fecal cortisol metabolites. *Physiol. Biochem. Zool.* 82: 291-301.

Bosson C.O., Palme R. & Boonstra R. 2013. Assessing the impact of live-capture, confinement, and translocation on stress and fate in eastern gray squirrels. *J. Mammal.* 94: 1401-1411.

Bradshaw D. 2003. *Vertebrate ecophysiology: an introduction to its principles and applications.* Cambridge University Press.

Campbell N. & Reece J. 2005. *Biology. 7th.* Benjamin Cummings, San Francisco.

Chelini M.M., Otta E., Yamakita C. & Palme R. 2010. Sex differences in the excretion of fecal glucocorticoid metabolites in the Syrian hamster. *J. Comp. Physiol.* 180: 919-925.

Dantzer B., McAdam A.G., Palme R., Boutin S. & Boonstra R. 2011a. How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. *Gen. Comp. Endocrinol.* 174: 124-131.

Dantzer B., McAdam A.G., Palme R., Humphries M.M., Boutin S. & Boonstra R. 2011b. Maternal androgens and behaviour in free-ranging North American red squirrels. *Anim. Behav.* 81: 469-479.

Dantzer B., McAdam A.G., Palme R., Fletcher Q.E., Boutin S., Humphries M.M. & Boonstra R. 2010. Fecal cortisol metabolite levels in free-ranging North American red squirrels: assay validation and the effects of reproductive condition. *Gen. Comp. Endocrinol.* 167: 279-286.

Eccard J.A., Fey K., Caspers B.A. & Ylönen H. 2011. Breeding state and season affect interspecific interaction types: indirect resource competition and direct interference. *Oecologia* 167: 1-11.

Franceschini C., Siutz C., Palme R. & Millesi E. 2007. Seasonal changes in cortisol and progesterone secretion in Common hamsters. *Gen. Comp. Endocrinol.* 152: 14-21.

Gartner K., Buttner D., Dohler K., Friedel R., Lindena J. & Trautschold I. 1980. Stress response of rats to handling and experimental procedures. *Lab. Anim.* 14: 267-274.

Haemisch A., Guerra G. & Furkert J. 1999. Adaptation of corticosterone-but not beta-endorphin-secretion to repeated blood sampling in rats. *Lab. Anim.* 33: 185-191.

Halle S. 2000. Voles—small graminivores with polyphasic patterns. In: Anonymous Activity patterns in small mammals, Springer, pp. 191-215.

Harper J.M. & Austad S.N. 2000. Fecal Glucocorticoids: A Noninvasive Method of Measuring Adrenal Activity in Wild and Captive Rodents. *Physiol. Biochem. Zool.* 73: 12-22.

Huhman K.L., Solomon M.B., Janicki M., Harmon A.C., Lin S.M., Israel J.E. & Jasnow A.M. 2003. Conditioned defeat in male and female Syrian hamsters. *Horm. Behav.* 44: 293-299.

Keckeis K., Lepschy M., Schöpper H., Moser L., Troxler J. & Palme R. 2012. Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs. *J. Comp. Physiol. B.* 182: 985-996.

Lepschy M., Touma C., Hruby R. & Palme R. 2007. Non-invasive measurement of adrenocortical activity in male and female rats. *Lab. Anim.* 41: 372-387.

Monclús R., Rödel H.G., Palme R., Von Holst D. & De Miguel J. 2006. Non-invasive measurement of the physiological stress response of wild rabbits to the odour of a predator. *Chemoecology* 16: 25-29.

Montiglio P., Pelletier F., Palme R., Garant D., Réale D. & Boonstra R. 2012. Noninvasive monitoring of fecal cortisol metabolites in the eastern

chipmunk (Tamias striatus): validation and comparison of two enzyme immunoassays. *Physiol. Biochem. Zool.* 85: 183-193.

Möstl E., Rettenbeacher S. & Palme R. 2005. Measurement of corticosterone metabolites in birds' droppings: an analytical approach. *Ann. N .Y. Acad. Sci.* 1046: 17-34.

Nováková M., Palme R., Kutalová H., Janský L. & Frynta D. 2008. The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (< i> Acomys cahirinus). *Physiol. Behav.* 95: 187-193.

Palme R. 2005. Measuring fecal steroids: guidelines for practical application. *Ann. N. Y. Acad. Sci.* 1046: 75-80.

Palme R. & Möstl E. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Zeitschrift fuer Saeugetierkunde (Germany)* .

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

Sheriff M.J., Dantzer B., Delehanty B., Palme R. & Boonstra R. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia* 166: 869-887.

Touma C. & Palme R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Ann. N. Y. Acad. Sci.* 1046: 54-74.

Touma C., Palme R. & Sachser N. 2004. Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. *Horm. Behav.* 45: 10-22.

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

Ylönen H. 1988. Diel activity and demography in an enclosed population of the vole Clethrionomys glareolus(Schreb.). *Ann. Zool. Fennici* 25: 221-228.

Ylönen H., Eccard J.A., Jokinen I. & Sundell J. 2006. Is the antipredatory response in behaviour reflected in stress measured in faecal corticosteroids in a small rodent? *Behav. Ecol. Sociobiol.* 60: 350-358.

Zeugswetter F., Neffe F., Schwendenwein I., Tichy A. & Möstl E. 2013. Configuration of antibodies for assay of urinary cortisol in dogs influences analytic specificity. *Domest. Anim. Endocrinol.* 45: 98-104.

TABLE 1   The course of the experiment and distribution of the test animals.

1. Habituation to the experimental cages • 5 days	All 48 animals (24 males and 24 females)	
2. Circadian fluctuation study • 72 hours	*Corticosterone*	*Testosterone*
	12 males and 12 females	12 males and 12 females
3. ACTH challenge • 72 hours	↓ 12 males and 12 females (same individuals as above)	↓ x
4. Radiometabolism study • 72 hours	↓ 6 males and 6 females (randomly selected from the individuals used above)	↓ 6 males and 6 females (randomly selected from the individuals used above)

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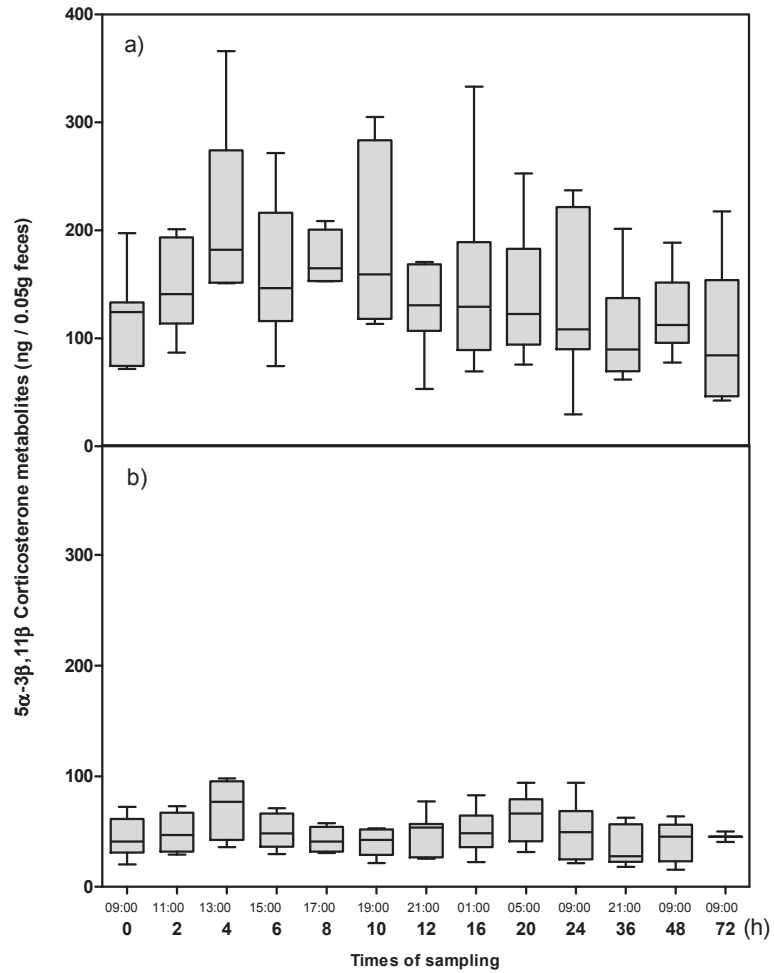
FIGURE 1   Excretion profile of immunoreactive CM in fecal samples of males (a) and females (b). Note the discontinuity in time in the X-axis due to the changes in the sampling interval after 12 hours.
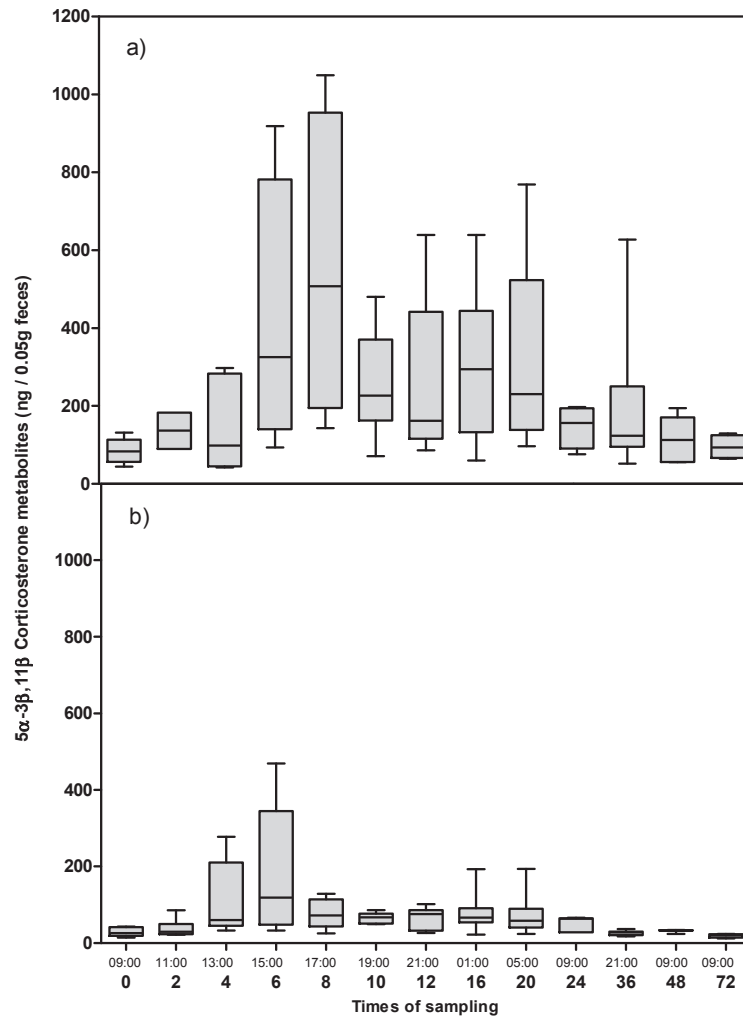
FIGURE 2   Excretion profile of immunoreactive CM in fecal samples in males (a) and in females (b) after ACTH injection. Note the discontinuity in time in the X-axis due to changing sampling interval after 12 hours.
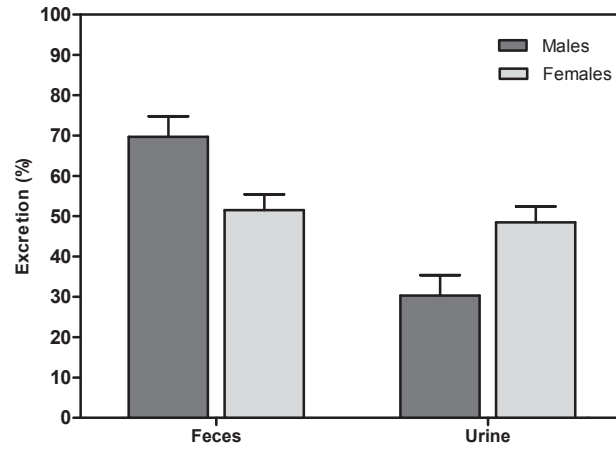
FIGURE 3   Amount of ³H-corticosterone metabolites (%) excreted via urine and feces in males and females.
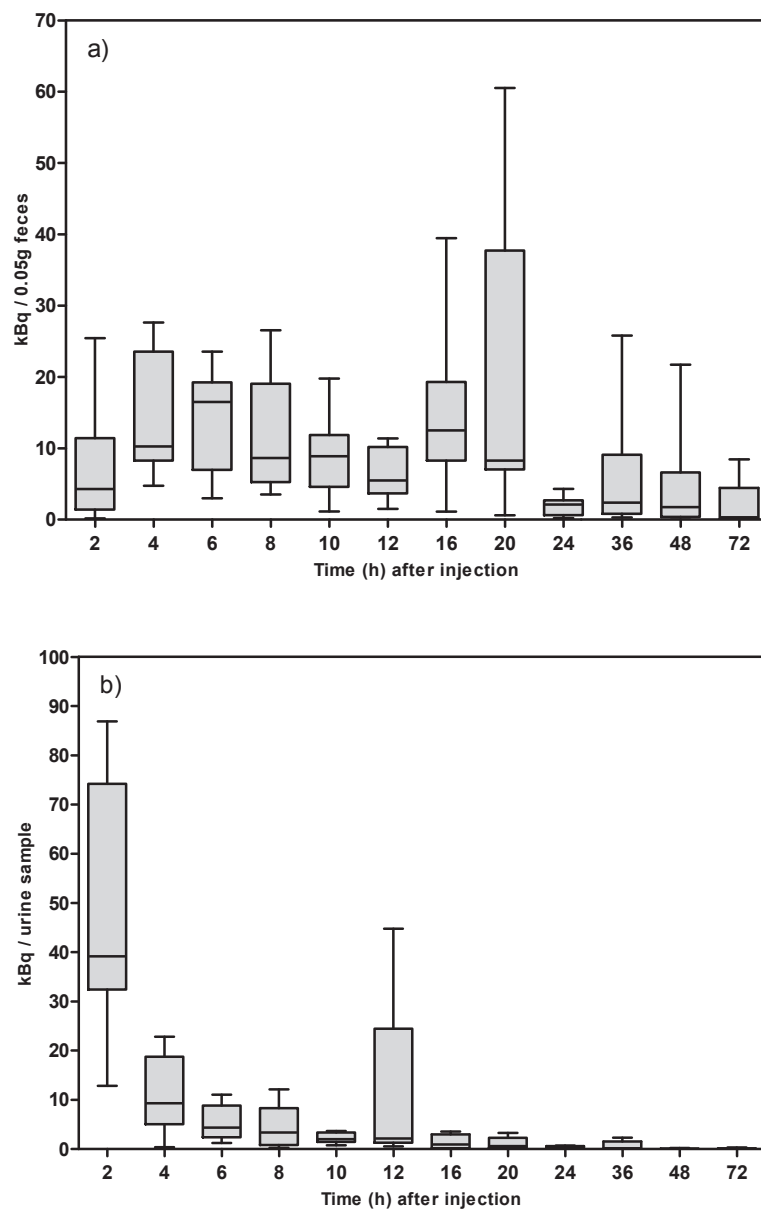
FIGURE 4   Time course of the excretion of ^{3}H-corticosterone metabolites
(kBq) in the feces (a) and urine (b) in males and females grouped together.
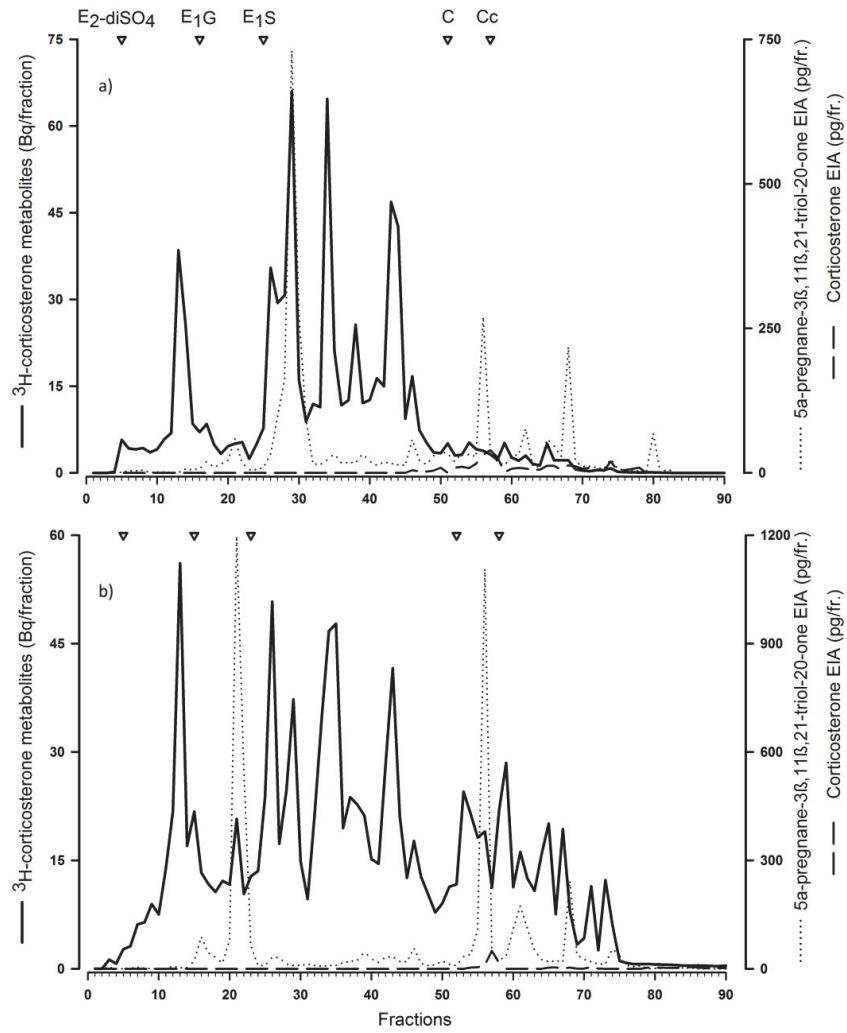
FIGURE 5   High performance liquid chromatographic (HPLC) separation of ³H-corticosterone metabolites in the feces of one male (a) and one female (b). Immunoreactivity was tested with two different enzyme immunoassays. Open triangles mark the approximate elution positions of respective standards (E₂-diSO₄ = 17β-oestradiol-disulfate, E₁G = oestrone-glucuronide, E₁S = oestrone-sulfate, C = cortisol, Cc = corticosterone).
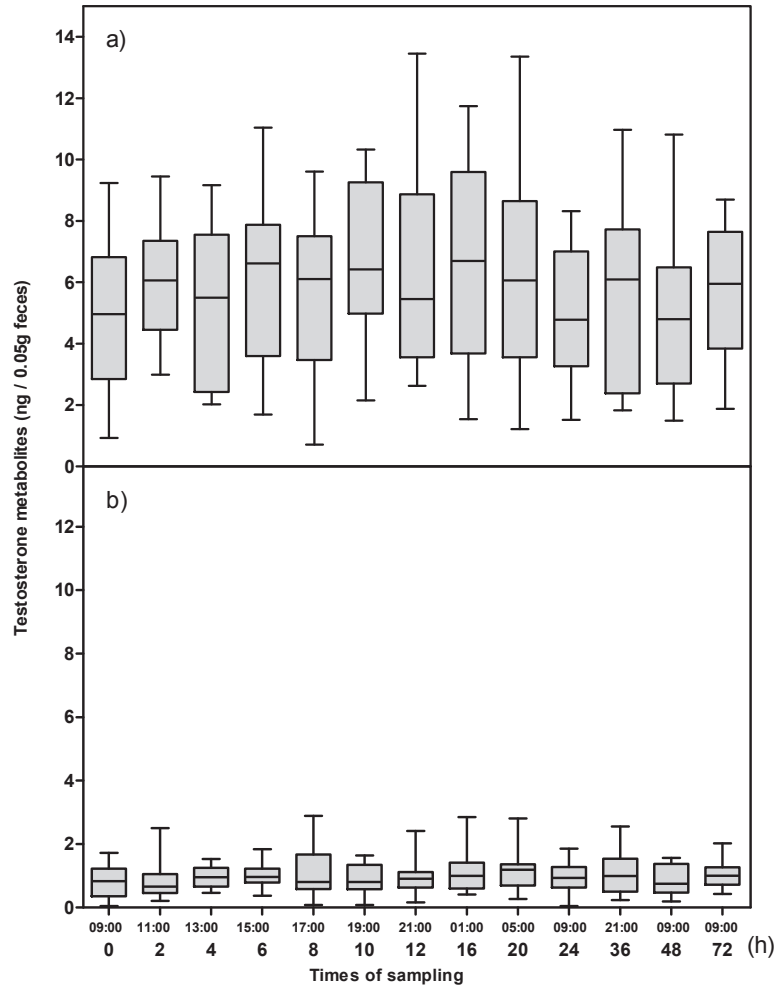
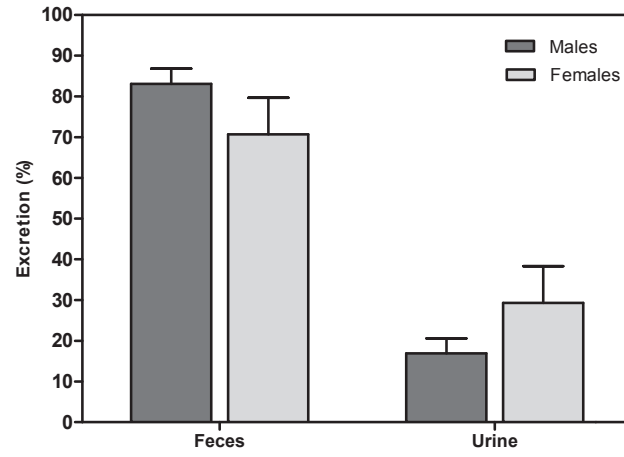FIGURE 6   Excretion profile of immunoreactive TM in fecal samples in males (a) and in females (b).

FIGURE 7   Amount of ^{3}H-testosterone metabolites (%) excreted via urine and feces in males and females.
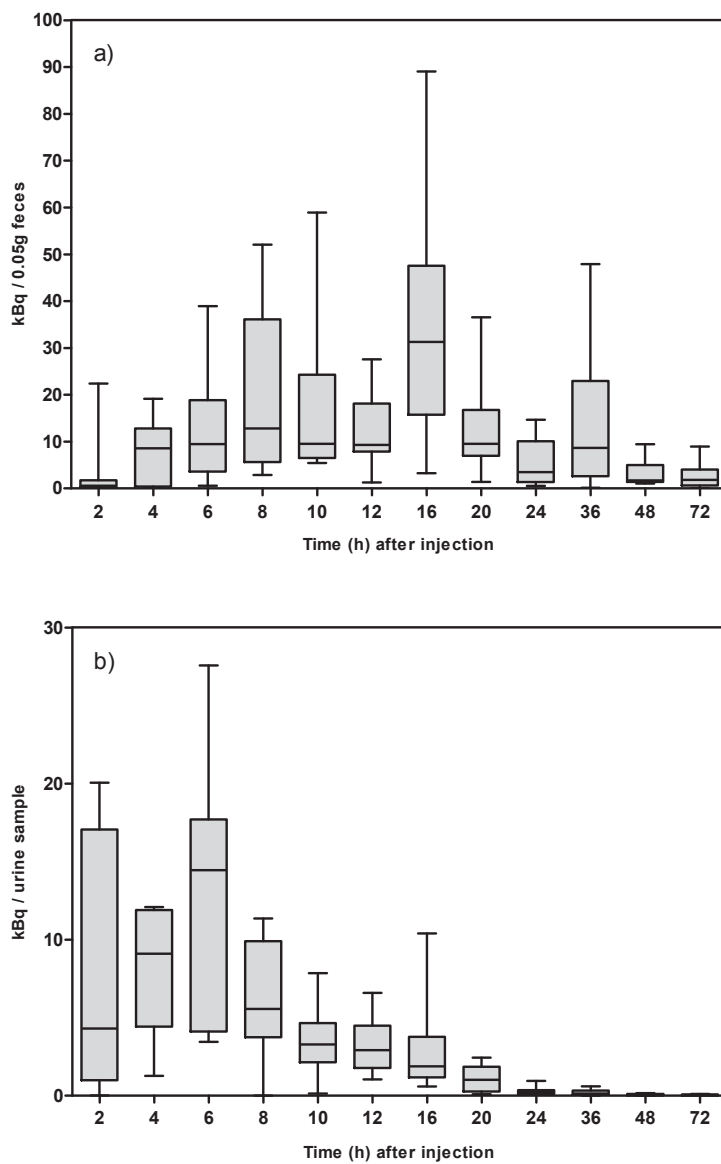
FIGURE 8   Time course of excretion of ^{3}H-testosterone metabolites (kBq) in feces (a) and in urine (b) in males and females.
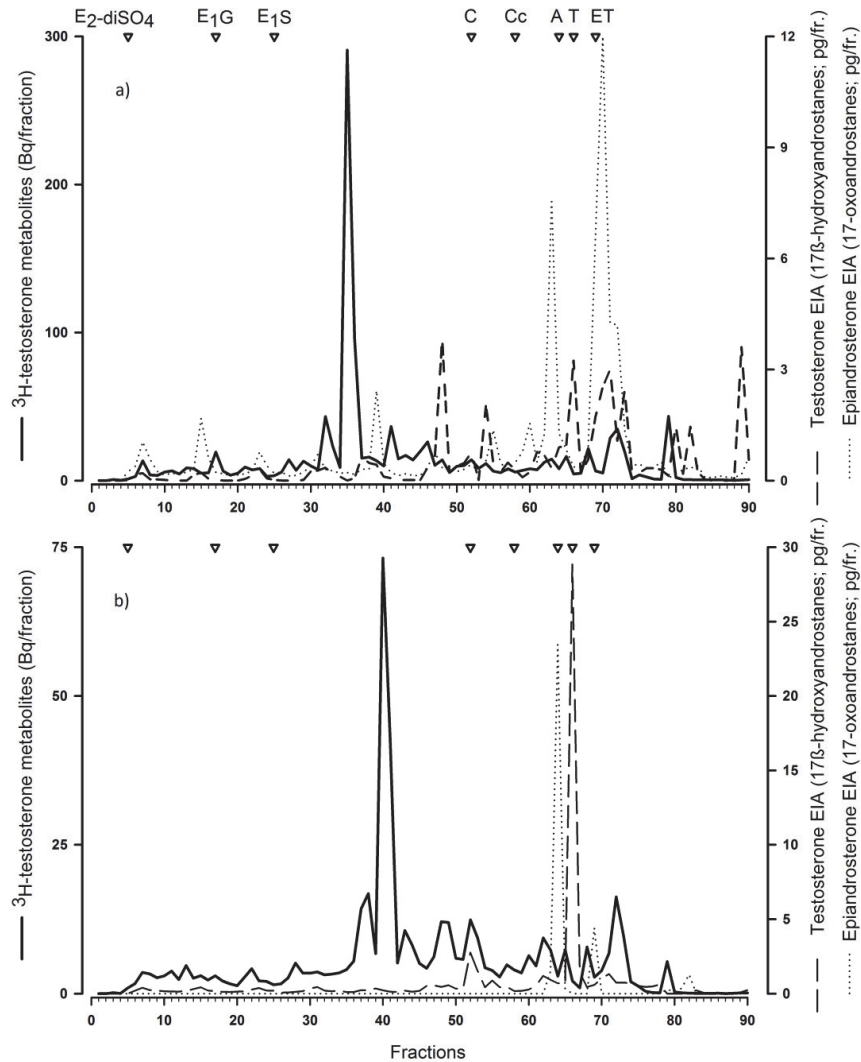
FIGURE 9   High performance liquid chromatographic (HPLC) separation of ^{3}H-testosterone metabolites in the feces of one male (a) and one female (b). Immunoreactivity was tested with two different enzyme immunoassays. Open triangles mark the approximate elution positions of respective standards (E$_2$-diSO4 = 17β-oestradiol-disulfate, E$_1$G = oestrone-glucuronide, E$_1$S = oestrone-sulfate, C = cortisol, Cc = corticosterone, A = androstenedione, T = testosterone, ET = epitestosterone).