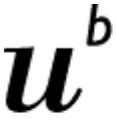
Impact of human disturbance on Alpine wildlife in winter: stress, activity and energetics in the endangered Black grouse *Tetrao tetrix*

Inauguraldissertation
der Philosophisch-naturwissenschaftlichen Fakultät
der Universität Bern

vorgelegt von
Marjana Baltić
von Kroatien

Leiter der Arbeit:
Prof. Dr. R. Arlettaz
Abt. Conservation Biology
Zoologisches institut
Universität Bern



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Black grouse *Tetrao tetrix* L. (photo by Stéphane Mettaz)

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Chapter One

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A non-invasive technique to evaluate human-generated stress in the Black Grouse

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Summary

The continuous development of tourism and related leisure activities is exerting a more and more intense pressure onto wildlife. In this study we tested and biologically validated a novel non-invasive method to measure stress in the Black grouse, an endangered, emblematic species of European ecosystems that is currently declining in several parts of its European range. A radiometabolism study and an ACTH challenge test were performed on four captive Black grouse (two of each sex) in order to get basic information about the metabolism and excretion of corticosterone and to find an appropriate enzyme-immunoassay (EIA) to measure its metabolites in the feces. Peak radioactivity in the droppings was detected within 1-2 hours. Injected ^{3}H-corticosterone was excreted as polar metabolites and by itself was almost absent. Among seven tested EIAs for different groups of glucocorticoid metabolites, a cortisone-EIA was chosen, as it cross-reacted with some of the formed metabolites and best reflected the increase of excreted corticosterone metabolites, after the ACTH challenge test. Concentrations of the metabolites from fecal samples collected from snow burrows of free-ranging Black grouse were within the same range as in captive birds. The described non-invasive method may be appropriate for evaluating the stress faced by free-living Black grouse populations in the wild, particularly in mountain ecosystems where human disturbance, especially by winter sports, is of increasing conservation concern.

Key words: Corticosterone metabolism, Non-invasive endocrine monitoring, Species conservation, *Tetrao tetrix*, Wildlife management

Introduction

In addition to habitat degradation, the intensification of human leisure activities exerts a negative pressure onto wildlife[1,2,3,4,5]. This is of particular concern as regards otherwise (e.g. through habitat degradation and fragmentation) already threatened and vulnerable species. Yet, until the recent development of appropriate analytical tools, it has remained difficult to quantify properly how human-generated disturbances affect animals' constitution, physiological condition and, ultimately, reproductive fitness. Not surprisingly most studies have therefore, until recently, focused on variations in time budgets and juvenile survival instead[1,3,6,7]. Rarely, however, could changes in time allocation to various behaviours and activities be associated with the actual additional physiological costs they entail. The emergence of non-invasive techniques for estimating stress in free-ranging animals opens new avenues for a proper quantification of the impact of human-generated stress onto wildlife[8,9,10,11,12,13,14,15].

The Black grouse (*Tetrao tetrix*), an emblematic game bird with great economic and cultural value, is endangered and declining in several parts of its Palaearctic distribution range[16]. Several authors have identified human activities and infrastructures as the probable main cause. This includes habitat loss and fragmentation[16], hunting[17,18], collisions with aerial cables and fences[19,20], as well as increasing disturbance through popular leisure activities such as winter sports[1,21,22]. Despite the knowledge, gathered from research on different species, that prolonged stress generated either by human or natural factors can have deleterious consequences on an individual[23,24,25], physiological constraints imposed by human activities are only poorly explored in the Black grouse[26,27]. In order to propose appropriate mitigation measures for this endangered species in those areas where leisure activities are a potential threat, a proper quantification of the impact of human-elicited stress appears a first necessary step. Additionally, however, as Black grouse are endangered, a non-invasive method, which does not alter or constrain birds' behaviour, is another prerequisite in any such study.

The first physiological response of an organism to different stressful stimuli is a cascade of hormone secretions starting with the release of catecholamines (epinephrine) within seconds after the stimulus from the adrenal

medulla, triggering within few minutes the hypothalamic–adenohypophysal–adrenocortical axis, which is followed by synthesis and secretion of glucocorticosteroids (corticosterone in the case of birds) from the adrenal cortex, as well as cytokines from cells of the immune system[23]. The blood sampling techniques usually applied in stress research are invasive. They are thus not convenient for the study of threatened, free-ranging animals such as the Black grouse[28]. Instead, non-invasive techniques for monitoring stress on free-living populations have therefore been developed extensively in the past years. In birds, feces are often used for this purpose, as they are easy to collect. However, it has to be taken into account that metabolites are excreted with a species-specific delay time of about a few hours in birds[9,10,11,29,30].

Until now, non-invasive methods for measuring adrenal activity by measuring fecal glucocorticoid metabolites by group specific enzyme-immunoassays (EIAs) have been mainly developed on domestic and captive animals for purposes of research on animal welfare[28,29,31] and behavioural ecology[10,12,32]. However, such methods have a great application potential in conservation biology also[9,13,15,22] . Metabolism and excretion of glucocorticoids differ between species and sometimes even between sexes and individuals within a given species[30,31]. Therefore, it is not possible to draw analogous conclusions from other bird species. The aims of this study were hence to get basic information about the metabolism and excretion of corticosterone, to characterize fecal metabolites of Black grouse, and to select an EIA for measuring the corticosterone metabolites (CM) in feces of free-ranging individuals. In addition, stability of the CM was tested under natural settings.

For this purpose, we performed first a radiometabolism study of corticosterone on birds in captivity. We also used the same birds for a biological validation of several EIAs by testing immunoreactivity of the CM excreted after adrenal stimulation with adrenocorticotropic hormone (ACTH). Yet, it should be noted that the stability of glucocorticoid metabolites in feces could be significantly affected by environmental conditions, due for instance to bacterial metabolism. This can give misleading results[33]. Both, to define a suitable sampling protocol of Black grouse feces in nature and to achieve accuracy in the hormone assay, we had to find out, first, if concentration of measured CM significantly changes in feces exposed to a manifold of winter environmental conditions, and, second, at which time intervals between defecation and

sampling of fecal pellets do these changes take place. This was performed by incubating feces in different time intervals and at temperatures above 0°C (below this threshold metabolic activity was assumed to be insignificant), which might occur in winter on sun-exposed snow surfaces. Also, we must stress that our radiometabolism study and the hormonal stimulation experiment for selecting the most appropriate EIA were carried out on captive birds that might have different stress reactions than free-ranging individuals, as well as distinct digestive tract size (caeca) and transit durations due to a different diet[34,35,36]. As corticosterone metabolism can be further affected by these factors, we analysed samples from free-living birds with the selected EIA to finally confirm the suitability of the method for assaying stress in wild Black grouse during the winter season.

Materials and methods

Birds and experimental set up

The permission for the animal experiment was given by the Department of Veterinary Medicine, canton Lucerne (Nr. 04/02). Two male and two female Black grouse, all 7 months old, were used in the experiments. Females originated from private raisers, males from Bern zoological garden. Between experiments, birds were kept in a large outdoor aviary at the Hasli Ethological Station of the Zoological Institute, University of Bern. Because we intended to apply this method on free-living birds during the cold season, we conducted laboratory experiments by the end of November 2002. At that time of the year, the birds had already reached adult plumage and body mass, whereas reproductive mechanisms, which could induce a stress state in displaying males[10], were not yet activated. During experiments, the birds were placed individually in cages with water and food provided *ad libitum*. Special cages (80 x 80 x 80 cm in size), with a double bottom, were constructed. The top of the cage as well as the back and one side walls were made out of green textile mesh (0.8 mm in diameter) to protect the birds against injuries due to a reduced space available. The front wall with the entrance, as well as one sidewall, consisted of wooden plates that isolated the birds from the researcher during sampling. The bottom of the cage comprised a wire-mesh floor (1 cm mesh diameter). This enabled droppings to fall underneath into a removable, exchangeable drawer. Cages and birds were

exposed to natural light and temperature conditions, but protected from precipitation.

Habituation of the birds to this set up lasted 4 days. By that time, they were feeding regularly and defecating normal droppings. As under natural conditions birds had at least two main feeding periods during the day[37,38] and defecated almost hourly 1-3 solid feces. To reduce the risk of a possible influence of different dietary compounds on digestion[34], only homogenous grouse food (article 872.4, Protector SA, CH-1522 Lucens) was provided.

Hormone administration and sampling pattern

Each bird was injected in the ulnar vein with 1.85 MBq of radiolabelled corticosterone ([1,2,6,7-^{3}H(N)]-corticosterone, specific activity: 76.5 Ci/mmol, NET-399, Perkin Elmer Life Sciences, Boston, MA), which was dissolved in 0.5 ml of a physiological solution (0.9% NaCl) containing 10% ethanol. Birds were injected between 8h00-8h15 am. Manipulation with each individual lasted less than 5 minutes.

Fecal samples were collected one hour before injection, to determine the background radioactivity, and after injection, once per hour during the following 24 hours. The feces were collected from the exchangeable drawers. They were frozen immediately at -22ºC until further analyses, and the drawers were cleaned with 70% ethanol and water after each sampling event.

In order to biologically validate different EIAs, the same birds were injected intravenously with 0.5 mg of adrenocorticotropic hormone (ACTH; Synacthen; Novartis Pharma AG, Basel, Switzerland). On the day before injection, the fecal samples were collected hourly during a total of 24 hours in order to get a control group (pre-treatment group). On the following day birds got the injection between 8h00 and 8h15 am and the fecal samples were again collected hourly during the next 24 hours (treatment group). During the third and fourth day, feces were collected early in the morning, at midday and in late afternoon in order to control for post-treatment levels (post-treatment groups I and II, respectively). All samples were immediately stored at -22ºC until analyses.

Extraction and characterisation of the excreted ^{3}H-corticosterone metabolites (CM)

A total of 0.5 g of each, well homogenized fecal sample was mixed with 3 ml methanol and 2 ml water and vortexed for 30 minutes. After centrifugation (2500 g; 10 min), aliquots (0.5 ml) of the supernatant (in duplicate) were transferred into scintillation vials (Article 6008117, Packard Instruments, Meriden, CT USA), each containing 6 ml scintillation fluid (Quicksafe, A, 100800, Zinsser Analytic, Maidenhead, UK). The radioactivity of each sample was measured (5 min) by a liquid scintillation counter (Tri-Carb 2100 TR, Packard Instruments, Meriden, CT, USA) with a quench compensation program. Radioactivity is expressed as kBq per g of feces.

In order to characterise the ^{3}H-corticosterone metabolites, samples containing peak radioactivity were extracted, the radioactive substances purified by a Sep-pak C_{18} cartridge and subjected to RP–HPLC (reverse-phase high-performance liquid chromatography) as described by Rettenbacher et al. [30]. Briefly, steroids were separated on a Novapak C_{18} column (3.9 x 150 mm, Millipore Corporation, Milford, MA, USA) with a methanol/water solvent. A linear gradient from 20-100% methanol with a flow rate of 1 ml/min was applied. A total of 96 fractions were collected (three per min). Radioactivity in an aliquot (50 µl) of each fraction was determined (Top Count; Packard Instruments, Meriden, CT, USA).

Immunoreactivity of CM and the biological validation of assays

An array of different, previously established EIAs was tested to select the best suited one for the Black grouse. Among the seven EIAs were a corticosterone-EIA[39], a tetrahydrocorticosterone-EIA[40], a 5α-pregnane-3β,11β,21-triol-20-one-EIA[31], an 11ß-hydroxyaetiocholanolone-EIA[41], an 11-oxoaetiocholanolone-EIA[42], a cortisone-EIA[30] and a so far unpublished 20β-dihydrocorticosterone-EIA. Aliquots of each HPLC fraction of males were measured with the different EIAs to check if radiolabelled metabolites were recognized. The EIA procedure was described in detail by Palme & Möstl[39] and Touma et al.[31]. The antibody of the 20ß-dihydrocorticosterone-EIA (working dilution 1:80'000) was raised against 20ß-dihydrocorticosterone-3-CMO:BSA in a rabbit. The label (20ß-dihydrocorticosterone-3-CMO-biotinyl-LC; 1:5'000'000) was produced as described by Möstl et al.[42]. The standard (20ß-dihydrocorticosterone) curve

ranged from 0.33 to 80 pg/well. Only the cortisone-EIA was applied on HPLC fractions of females, but the later three EIAs, which were able to detect significant amounts of immunoreactive substances in the HPLC fractions of males, were used for the analyses of the samples from the stimulation experiment (ACTH) of both sexes.

Stability of CM in the feces

In order to optimise the collection of fecal samples from free-living Black grouse, it was important to know whether and how the concentration of metabolites changes with time, especially when ambient temperature increases above zero and allows activation of fecal bacteria. We did this with captive birds, two males and one female, from which we collected feces on the third day (post-treatment control group II, see above) after the ACTH injection. All feces excreted by a single bird (ca 20 g) were pooled, homogenized, and divided into 4 equal sub samples. A sub sample was frozen immediately at –22°C (control), whilst the three other samples were incubated in a fridge at 6-7°C for 24 h, 48 h and 72 h, respectively. Temperature in the fridge was set up as the highest measured temperature in igloo, i.e. 6.5ºC (unpublished personal data). From each sub sample 10 aliquots of 0.5 g each were extracted and the concentration of the metabolites measured with the cortisone-EIA as described above.

Concentrations of CM in feces from free-ranging birds

In a preliminary field experiment, amounts of CM present in free-living birds were measured in order to get preliminary information on interindividual and intraindividual variances. We collected samples from the snow igloos of four free-living Black grouse males, in February 2003, at Verbier and Les Diablerets (south-western Swiss Alps). The birds were flushed from their diurnal igloos early in the afternoon and fecal material accumulated within igloos (9-15 separate droppings each) was collected. In order to gather information about within igloo variance of CM concentrations of an individual bird, each dropping was analysed separately. The cortisone-EIA, which gave the best results in the ACTH challenge test, was selected for further analysis of the feces from the free-living birds. All feces were extracted as described above (5 ml of 60% methanol) and an aliquot (diluted 1:10) of the supernatant analysed in the EIA.

Statistical analyses

Results of the ACTH test (biological validation) of the three EIAs, which cross-reacted significantly with radioactive metabolites, were analysed by an ANOVA standard least square fit model. We tested for the following effects and interaction term: individual bird (n = 4), treatment (vs. control day, i.e. data from the day prior to ACTH administration), bird*treatment.

The within-individual (i.e. within igloo fecal sample) and among-individual (between igloos) variation in CM concentrations of the feces from free-ranging black grouse males was analysed by one-way ANOVA after controlling for variance homoscedasticity (Levene's test). All statistical analyses were performed with JMP 4.04 (SAS Institute Inc. 1989-2001). Test rejection probability levels were set throughout at 5%.

Results

Excretion and characterisation of the ^{3}H-CM

The main portion of radioactivity was quickly excreted. Peak concentrations (75 to 139 kBq/g feces) were reached after one (one male and one female) or two (the other two animals) hours (Fig. 1). Radioactivity decreased almost continuously (only one male had a second, somewhat smaller peak after 5 h) afterwards. When sampling was stopped 24 h after the injection, radioactivity was low, but background levels were not yet reached.

Injected ^{3}H-corticosterone was heavily metabolised, as demonstrated by HPLC separation of CM of the peak radioactivity samples of the four individual birds (Fig. 2). Three to four main metabolites were present, all eluting between fractions 20 and 45, thus resembling conjugated, or polar unconjugated steroids. Males had more polar metabolites if compared with females. In all samples only small amounts, if at all, of unmetabolised corticosterone could be detected. In the HPLC fractions significant amounts of CM could be measured with three of the seven EIAs tested (Fig. 2), i.e. concentrations were higher than the detection limit of the respective EIAs. The cortisone-EIA measuring metabolites with a common 3,11-dione structure yielded the highest amounts of immunoreactivity. The most prominent metabolite peaked at fractions 31/32. The 11-oxoaetiocholanolone- and the 11ß-dihydrocorticosterone-EIA measured only smaller amounts of immunoreactivity.

Biological validation of the EIAs – ACTH challenge

The concentrations measured with the cortisone-EIA (3,11-dioxo-CM) differed significantly between the experimental groups (ANOVA, df = 1, F = 16.41, p < 0.0001), but no difference among individuals (ANOVA, df = 3, F = 1.933, p = 0.128) was found, although males tended to have higher basal values. A p*ost hoc* test showed that it was due to differences between the treatment group (i.e. data obtained during the first 24 h after adrenal stimulation) and the pre-treatment, on one side, and the post-treatment I and II control groups, on the other side (Dunnett's test). Note that 24 h after injecting ACTH, the concentration of the CM had returned approximately to the levels recorded before the experimental treatment (post-hoc Dunnett's test, not significant).

During the pre-treatment day (pre-treatment control group), the mean (±SE) concentration of CM in droppings of all four birds was 454 ± 31 nmol/kg feces (Fig. 3). On the second day, after the ACTH injection at 8h-8h15 am, the concentration of CM reached a maximum (3 to 12 µmol/kg) during the first three hours, which represents a 13 fold increase in comparison with control, pre-treatment baseline values. Within the following four hours, the concentration decreased again to 594 ± 25 nmol/kg, a level that remained more or less constant over the next 16 hours. On the third day of the experiment (post-treatment group I), concentrations returned to levels similar to pre-treatment (453 ± 41 nmol/kg feces) and remained similar on the fourth day (post-treatment group II; 438 ± 56 nmol/kg).

The four birds reacted differently to the ACTH injection. The strongest response (24 fold magnitude in comparison with control values) was observed in female 2, but it should be mentioned that this bird showed the lowest mean baseline concentration of CM. The highest peak of metabolites, amounting to 12 µmol/kg feces, was found in the male, which already exhibited the highest average concentration of metabolites in the pretreatment control group.

As with the cortisone-EIA, there was a significant difference in the concentrations of the CM measured with the 11-oxoaetiocholanolone- and the 11ß-dihydrocorticosterone-EIA between the pre-treatment control group and the treatment group during the 48 hours following the ACTH injection (ANOVA, 11-oxoaetiocholanolone-EIA: df = 1, F = 24.28, p < 0.0001; 20β-dihydrocorticosterone-EIA: df = 1, F = 57.49, p < 0.0001), although no

prominent peak was recognizable. In addition, concentrations of these CM were 5 to 20 times lower than the 3,11-dioxo-CM measured with the cortisone-EIA and showed statistical differences among individuals (ANOVA, 11-oxoaetiocholanolone-EIA: df = 3, F = 41.13, p < 0.0001; 20β-dihydrocorticosterone-EIA: df = 3, F = 26.61, p < 0.0001).

Stability of CM in the feces

After incubation of feces at 6-7ºC in a fridge, a slight decrease of CM (by 16%) was noticed after 24 hours, which however was not statistically significant (360 nmol/kg vs. 430 nmol/kg for the control sample, Fig. 4). Concentrations remained close to that level after 48h and 72h (average decrease by 21% and 17%, respectively, from the control sample).

Concentrations of CM from feces of free-ranging birds

Concentrations of CM in free-ranging Black grouse assayed by the cortisone-EIA ranged from 62 to 1993 nmol/kg feces (Fig. 5). There was a significant interindividual variation in metabolite concentrations (ANOVA, $F_{[3,43]} = 14.852$, I < 0.0001), but within igloo variances did not differ significantly (Levene's test, $F_{[3,43]} = 1.048$, p = 0.381). This range of concentrations was similar, or somewhat higher than that of captive birds on the control day prior to the ACTH challenge experiment, but much lower than concentrations induced by ACTH (Fig. 3) assayed with the same EIA.

Discussion

In this study, we successfully tested and biologically validated a non-invasive method for evaluating adrenocortical activity in the Black grouse. This offers the novel possibility to quantify the level of stress, for instance induced by human disturbance, in free-ranging populations of this endangered species, as feces can easily be collected from snow burrows in winter. In line with previous investigations, we could demonstrate that assaying corticosterone metabolites (CM) by group specific enzyme immunoassays (EIAs) gives an accurate picture of the adrenocortical activity[28]. This is the first time that a non-invasive technique is described for evaluating disturbances, via corticosterone metabolites measured by EIA in fecal material of a species of Phasianidae.

Birds' excreta consist of an inhomogeneous cloacal mixture of urine and feces. Steroid metabolites are excreted in urine and feces at different time intervals, first in urine, and some hours later in feces[30,31,42]. In the Black grouse, as in chicken[30], a first peak of radioactivity appeared within the first two hours after injecting ^{3}H-corticosterone (Fig. 1). This corresponded to an initial period when most excreta in all four birds were rather liquid, containing a large part of urine components. Yet, contrary to what was found in other studies[11,30,44], a second peak, reflecting excreted fecal metabolites, roughly corresponding to the timing of gut passage, was not detected in Black grouse (except in one male), where the concentration of metabolites decreased almost continuously. The reason for this may be a more expressed urinary excretion, thus concealing the smaller amounts of fecal metabolites or some mixing of excreta in the cloaca.

Our reverse-phase high-performance liquid chromatography (HPLC) analysis of ^{3}H-corticosterone metabolites from feces of two captive males and females demonstrated that corticosterone is heavily metabolised mainly to polar metabolites, whereas corticosterone itself was almost absent. This corresponds to the findings of Goymann et al.[11] in European stonechats, Carare et al.[32] in Great tits, and Rettenbacher et al.[30] in chicken. Altogether, three to four prominent peaks, probably representing conjugated CM, were present in all four Black grouse individuals. There were also some apparent sex differences in the pattern of metabolites formed, similar as shown for other vertebrate species[30,31].

Only three out of seven tested, group specific EIAs cross-reacted significantly with the ^{3}H-CM present in the HPLC fractions (Fig. 2). In order to choose the best suited one to assess adrenocortical activity, all those three EIAs were biologically validated. This was achieved by the ACTH challenge test. Blood samples were not taken, as corticosterone concentrations in plasma are known to correlate well with the concentration of metabolites in the feces[45,46]. As we were interested in the pattern of CM in the droppings, it was important to avoid confounding effects of the stress experienced by the blood sampling procedure itself. In addition, we did not want to apply an invasive, frequent blood sampling regime to the endangered birds.

Injection of ACTH resulted in a distinct increase in measured 3,11-dioxo-CM concentrations in all four birds. After the initial peak, taking place during the first 2-3 h after ACTH administration, there was a rapid decrease of CM values within 4 h. Peak concentrations were approximately 13 times higher than

baseline values (Fig. 3). This increase was more pronounced than in chicken[30] and makes it more probable that also some less stressful events can be monitored by fecal analysis.

Although sex differences were observed in the pattern of formed ^{3}H-CM, there were no statistically significant differences in measured levels of 3,11-dioxo-CM. This is another advantage of the cortisone-EIA, as the gender of an animal can be neglected in comparative analyses of stress levels faced by birds under various environmental conditions. The other two assays (11-oxoaetiocholanolone and 11ß-dihydrocorticosterone-EIA) were found to be unsuited for evaluating stress properly, as the measured concentrations were much lower, no distinctive peaks could be recognized after ACTH injection and pronounced individual differences were observed.

As Washburn and Millspaugh[33] and Morrow et al.[47] showed for «even-toed» ungulate feces, environmental conditions, particularly moisture in combination with higher temperatures can significantly affect CM degradation in the feces, as they would favour the activity of bacteria. This could be a serious source of bias in the quantification and interpretation of stress levels. One advantageous trait of Black grouse is that they use to roost in snow burrows in winter, in which they defecate. This enables the collection of fecal samples, which are so to say naturally stored in optimal temperature conditions, which remain in any case lower than in our incubation experiment at 6-7°C, where degradation was only slight.

Another question is whether there is variation in CM between droppings of the same individual within a short time. This could for example be due to a diurnal rhythm. In our preliminary field experiment, the concentrations of CM taken from samples of free-ranging birds varied considerably within the individual. However, the mean of the individuals still differed significantly. Therefore, it is advisable to take all droppings from a snow igloo and homogenize them before analysis. We think, that this is the best way to characterize the level of CM over the time the feces were excreted.

We conclude that measuring corticosterone metabolites from feces of Black grouse with the cortisone-EIA provides a suitable, novel tool for quantifying non-invasively adrenocortical activity and thus stress in free-ranging Black grouse populations. This will enable to investigate properly the levels of stress, acclimation and facilitation actually faced by this endangered bird species,

especially in mountain habitats, where increasing leisure activities might potentially represent a serious additional source of threat to the fauna in general. In the future, we may envision to model tolerance thresholds towards human disturbance on Black grouse populations. This might be an essential step for proposing sound, targeted conservation measures to mitigate the impact of man on that emblematic species.

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Figure legends

Fig. 1. Excreted radioactivity (kBq/g feces; min., max., median) in the droppings of four Black grouse birds after ^{3}H-corticosterone administration. Animals were injected intravenously between 8h00-8h15 am (0 h, arrow).

Fig. 2. Immunoreactivity (nmol per fraction) of ^{3}H-corticosterone metabolites (Bq per fraction) evaluated by reverse phase high performance liquid chromatography (RP-HPLC) with three different EIAs (cortisone-, 11-oxoaetiocholanolone- and 11ß-dihydrocorticosterone) in two male and two female Black grouse. Elution positions of corticosterone, cortisol, 17β-oestradiol-disulfate (E_2β-diSO_4), oestrone-glucuronide (E_1G) and oestrone-sulfate (E_1S) are marked. A gradient solvent system with a water/methanol ratio changing from 20 - 100% was applied.

Fig. 3. Concentrations (min., max., median) of corticosterone metabolites (3,11-dioxo-CM; nmol/kg feces) before (upper panel: pre-treatment group) and after stimulation with ACTH (lower panel: treatment group), measured by a cortisone-EIA in two male and two female Black grouse. Injections took place at 8h00-8h15 am.

Fig. 4. Concentrations (mean ± SE; n = 3) of corticosterone metabolites (3,11-dioxo-CM; nmol/kg feces) after incubation at 6-7ºC for 24, 48 and 72 h, respectively. Controls stem from samples that were immediately frozen at -22°C.

Fig. 5. Boxplots of 3,11-dioxo-CM concentrations (nmol/kg feces) from samples from the snow burrows of four free-ranging Black grouse males in February 2003.

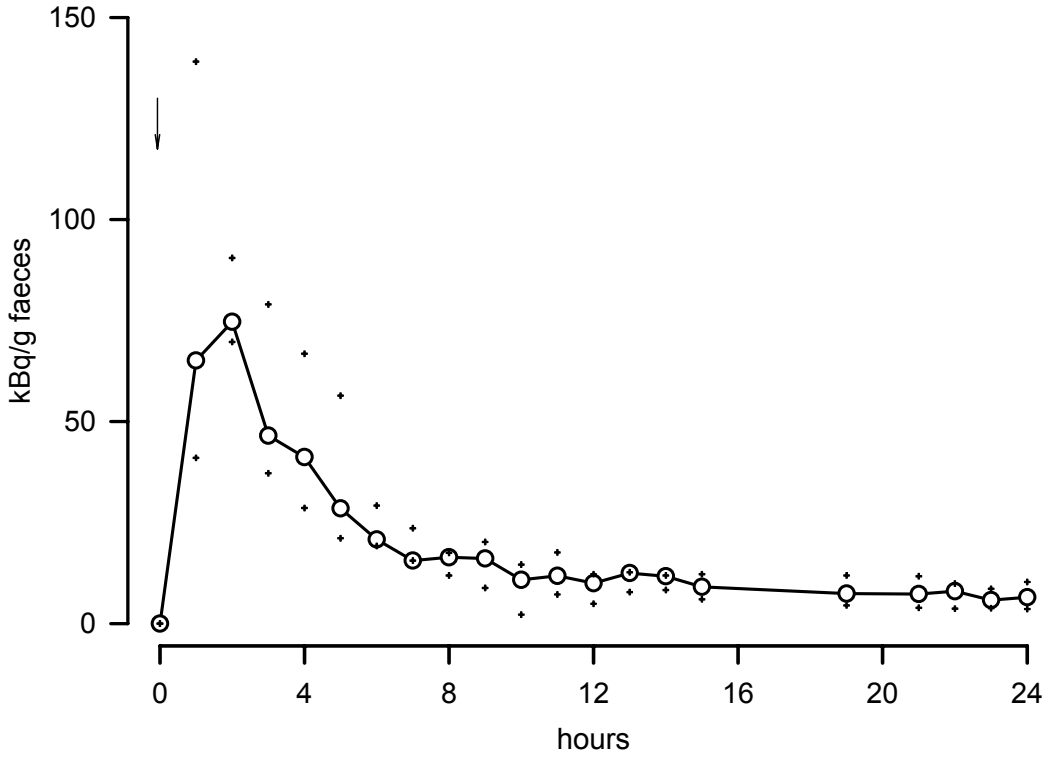
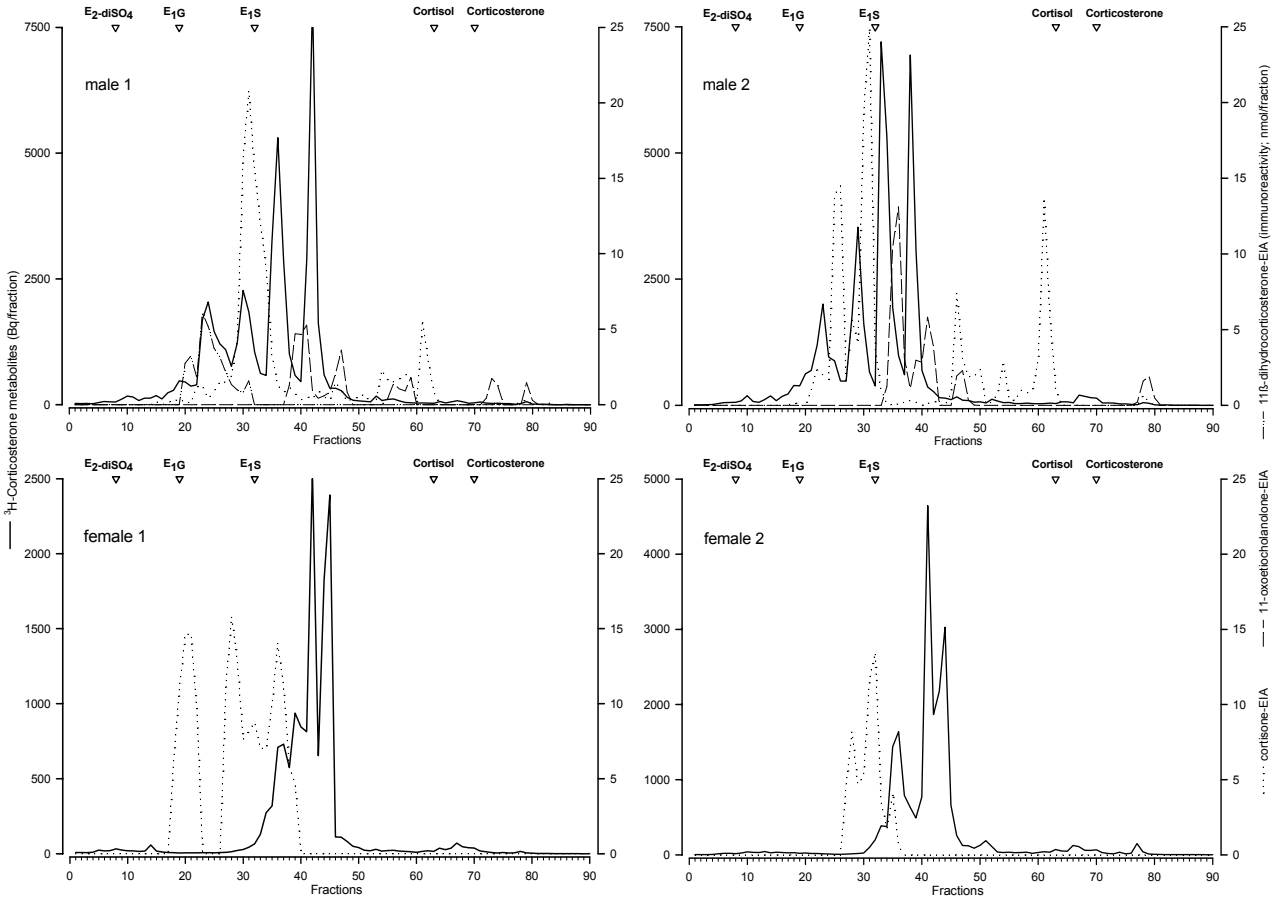


Fig. 1. Baltic et al.



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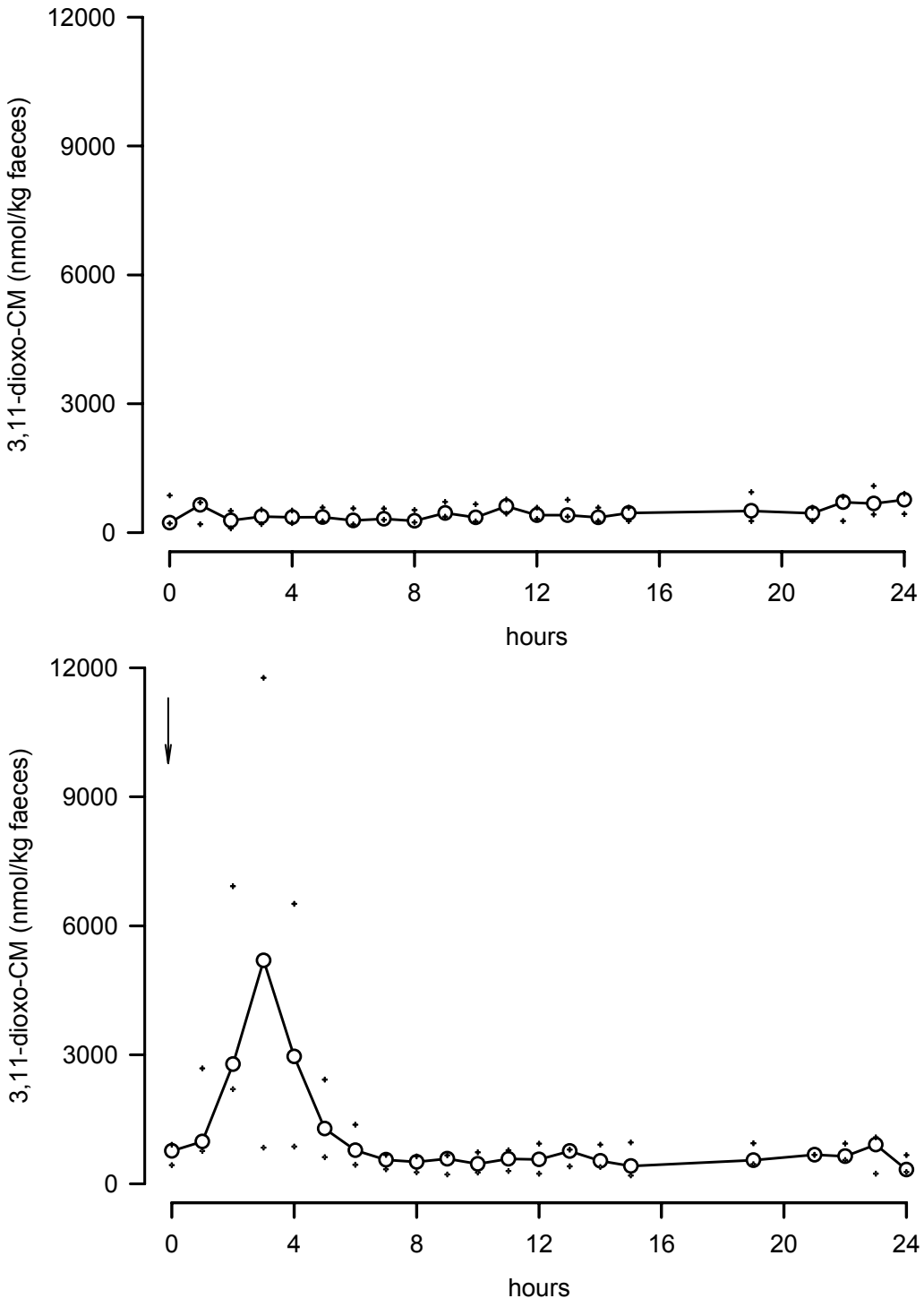

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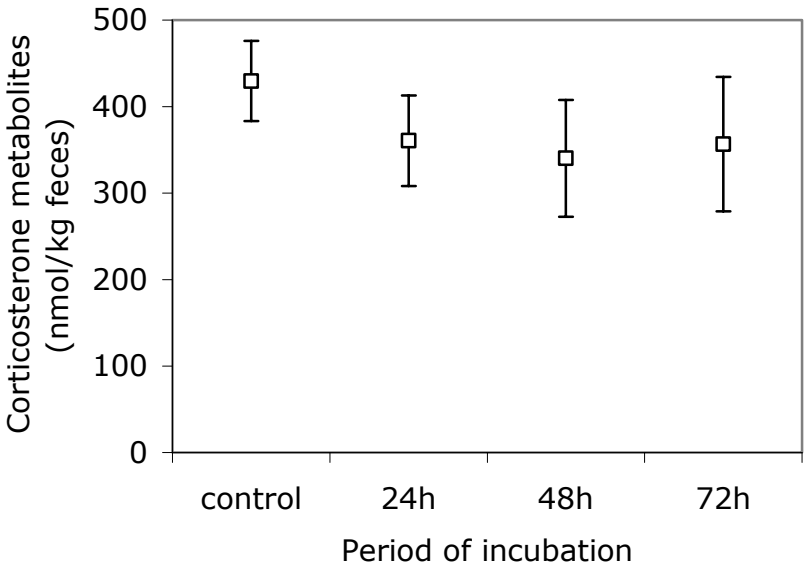

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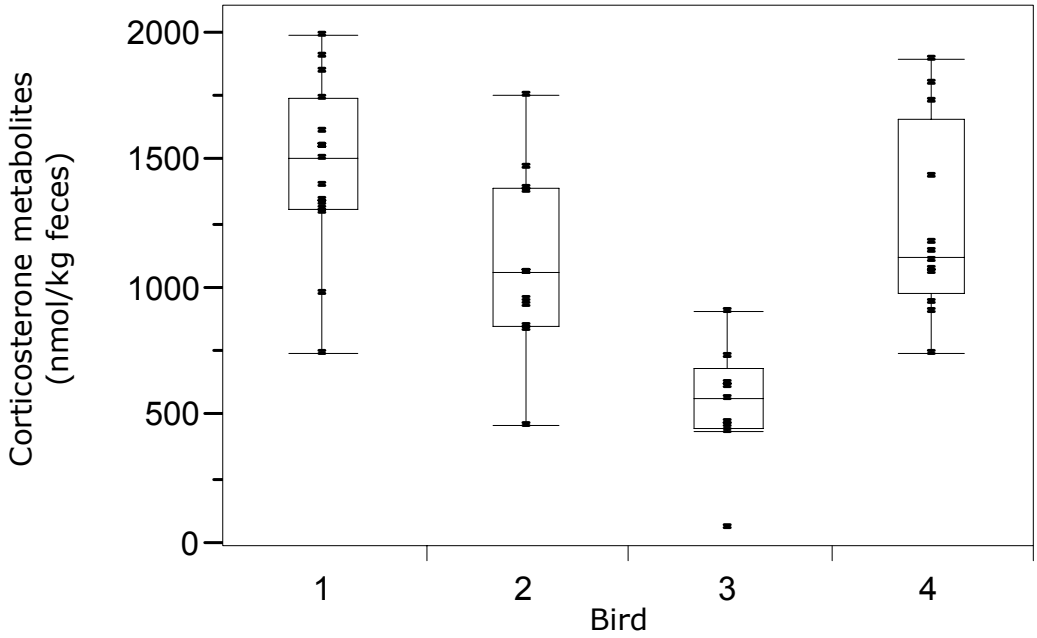

Fig. 5. Baltic et al.

Chapter Two

Spreading free-riding snow sports represent a novel source of threat for wildlife

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Manuscript

Spreading free-riding snow sports represent a novel source of threat for wildlife

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Key words: Alpine ecosystems, Black grouse, Species conservation, Stress, *Tetrao tetrix*, Winter touristical industry

Human-generated stress onto wildlife, through continually developing outdoor recreational activities, is of increasing conservation concern as it often adds to other factors already affecting negatively the dynamics of vulnerable populations[1]. It remains unclear, however, to which extent rapidly spreading free-riding snow sports, as a result of the intensifying winter tourism industry, actually elicit detrimental stress upon wildlife, with associated fitness and survival costs. Using a non-invasive method[2-5] we evaluated the levels of physiological stress induced by free-riding snow sports onto free-ranging Black grouse, a declining species of Alpine ecosystems[6]. The concentration of stress hormone metabolites was measured in droppings collected from birds' snow burrows[7]. In radiomonitored birds actively flushed from igloo once a day, during four consecutive days, basal stress increased continuously from control day throughout to the end of experiment. A comparison of stress levels among habitats with different degrees of disturbance by skiers and snowboarders also revealed higher stress in birds in disturbed vs. undisturbed habitats. This study shows that disturbance through free-ride skiing and snowboarding actually leads to stress and represents a new serious additional threat for wildlife.

Wild animals have to cope not only with predictable characteristics of the environment, such as seasonal changes in climate and resource availability, but also with a variety of unpredictable events, including human disturbance which tends to increase in most ecosystems worldwide[1,8,9]. All perturbation factors have the potential to cause stress responses, resulting in an activation of the hypothalamo-pituitary-adrenal axis, leading to an increased glucocorticoid production[9-12]. The aim of that mechanism is to adjust the physiology and behaviour to the prevailing environmental conditions, including unpredictable events. It allows to efficiently cope with adverse circumstances, so as to minimize stress, i.e. to ensure survival and a progressive return to «normal» life[13]. If stress persists or is repeated over time, an animal may remain physiologically affected and may suffer from a variety of symptoms[13]. These may eventually lead to a reduced individual fitness and, if stressful events attain many members of a population repeatedly, to a progressive population decline[14]. Populations of wild animals which are already classified as vulnerable, due to global environmental changes and local habitat alteration, face today new

potential additional threats conveyed by a burgeoning human demography, such as the growing touristical industry[15,16]. The popularisation of extreme sports impels more and more people to enjoy and test their sport skills outside traditional touristical zones, with nature being merely used as a décor for such trendy activities. Winter fun sports develop rapidly, with little consideration for the fate of organisms inhabiting alpine habitats[1].

In the European Alps, where most winter outdoor touristical activities aggregate across the continent, several wild animal species are currently undergoing alarming declines. For instance, the International Union for the Conservation of Nature (IUCN) assumes that factors responsible for the decline of the Alpine Black grouse are linked to the spreading and intensifying of winter sports. Free-riding skiers and snowboarders disturb resting birds, whilst skilifts, installed mostly around the timberline zone (1800-2300 m altitude), deteriorate primary Black grouse habitat[6]. Black grouse can survive in fragmented habitats among ski resorts, at least as long as skiers and snowboarders remain on ski pistes (pers. obs.). Yet, Black grouse are increasingly exposed to irregular, unpredictable disturbance by free-riding skiers and snowboarders who cross their habitat and inadvertently flush them from their snow burrows. Roosting in igloos is a crucial anti-predator[17] and energy-saving strategy[18]. The latter enables Black grouse to cope with adverse weather conditions, in particular low winter temperatures. Flushing from igloo not only elicits costly, abrupt escape flights, but it also provokes costly «outdoor» stays, affecting birds' finely tuned winter energetic balance[18]. A problem, which is still poorly understood, is whether human winter disturbance evokes a physiological stress response – with associated energetic expenditures – leading to an increase of circulating glucocorticoids, and whether repeated disturbance may lead to a chronic stress state. To answer this question, we monitored the main avian glucocorticoid, corticosterone, which reflects adrenal activity. The concentration of the metabolites of this stress hormone were measured from faecal material, i.e. non-invasively and retrospectively [2,7,19]. Faeces are deposited twice daily and every time in a new igloo since Black grouse burrow two igloos per day, i.e. one after each feeding session. Faecal material in igloos is naturally stored in environmental conditions (<0°C) that prevent degradation of hormones by enzymes, bacteria and sun radiation[7]. In a field experiment, we first actively flushed radiotagged Black grouse from their snow burrows once a day, during

four consecutive days, and collected droppings from igloos for an assessment of faecal corticosterone metabolites (FCM). This enabled us to test whether stress increased after initial disturbance, and whether there was an additive effect over the course of the experiment. Secondly, we compared FCM concentrations among 32 sites that showed various degrees of disturbance by free-riders in order to see if basal stress was more acute in habitats frequently visited by skiers and snowboarders.

In the field experiment, concentrations of FCM increased continuously from the control day throughout to day 3 of experiment (Fig. 1a), with day being a significant factor (Wald test, $\chi^2 = 4.2$, df = 1, p = 0.04), but not its quadratic term ($\chi^2 = 1.3$, df = 1, p = 0.26). The non-significance of the squared term indicates that, during the experiment, no plateau in FCM levels was reached, pleading for a short-term fully additive effect of reiterated disturbances on stress. Using the model with only day as fixed term, we predict that FCM concentrations increased daily by 146.1 $\pm$ 75 nmol/kg (mean ± SE), which corresponds to an increase of ca 50% between control day (baseline stress) and day 3 of the experiment.

In the comparative analysis, FCM concentrations differed significantly between habitats with various levels of human impact (Wald test, $\chi^2 = 7.5$, df = 2, p = 0.024; Fig. 1b); there was also a significant effect of the factor site (nested within category), reflecting some variation among sites within a given habitat category (likelihood ratio test: $\chi^2 = 15.9$, df = 1, p < 0.001). Pairwise comparisons showed that birds in disturbed habitat had significantly higher concentrations of FCM (ca 30-40% more) than habitat with no or very limited (0-1 trace) human disturbance (contrast test: $\chi^2 = 7.0$, df = 1, p = 0.008), whereas FCM concentrations did not differ between habitats with moderate vs. high human disturbance (contrast test: $\chi^2 = 0.5$, df = 1, p = 0.48).

Combining experimental and comparative data, this pilot study demonstrates for the first time that disturbances elicited by free-ride skiing and snowboarding actually provoke a dramatic stress response in a threatened species of the Alpine fauna. Since flushing of Black grouse and faecal sample collection took place every day in early afternoon (14-15 h), birds apparently remained in a stress state up to a minimum of 16 h after a flushing event. Collected faeces were defecated in igloos after early morning feeding and therefore igloo samples did not contain faeces excreted 1-3 h after flushing, i.e.

they were likely not containing the extremely high peaks of corticosterone concentrations that occur right after a stressful event, as demonstrated earlier[7]. The values recorded here might therefore be considered as minimum estimates. The outcome of the experiment is particularly striking as our disturbance was restricted to one single flushing per individual bird and day, which does not necessarily represent a worst case scenario since close to some ski resorts Black grouse can potentially be disturbed several times a day. As shown by our results, repeated disturbances, particularly occasional unpredictable events, if occurring over successive days may clearly induce stress, and, therefore, potential long-lasting physiological effects, as supported by our comparative analysis of FCM along the disturbance gradient. FCM concentrations did not reach a plateau during the duration of our flushing experiments, pointing to a clear short-term additive effect of disturbance. Further studies are necessary to evaluate whether physiological adaptations to repeated stress are plausible in the mid term.

Black grouse which are disturbed from their resting igloos in winter are not only impaired by the physiological costs linked with sudden escape flights and a mounted stress response[12], as established here, but they also face other additional costs and risks. First, as long as they stay outside snow burrows they have to thermoregulate more intensively, as they do no longer benefit from the temperature buffer of igloos; this might become especially critical at low ambient temperatures. Second, when they leave their igloos after disturbance, they either rest on trees for a while or immediately start snow-burrowing elsewhere: this renders them more conspicuous to predators[18]. All these factors, either acting singly or conjugatedly, are likely to ultimately affect birds' winter energetic balance, physiological condition, immunological competence, survival and reproductive ability[13,20]. Future investigations ought now to quantify the implications of disturbance by free-riding snow sports upon wildlife fitness and survival[21]. This will be a necessary step to define tolerance thresholds towards spreading and intensifying winter outdoor sports, so as to mitigate any detrimental human-induced impact and to promote secure wintering zones for wildlife.

Methods

Field experiment

Three Black grouse males were mist-netted at lekking places, nearby two ski resorts in the southwestern Swiss Alps (Verbier 46°06 N, 07°15 E and Les Diablerets, 46°20 N, 07°07 E) in May 2002. They were tagged with 16 g neck-laced radio transmitters equipped with an activity sensor (Holohil Systems Ltd, Carp, Canada), under license of the Wildlife and Game services of Valais and Vaud. Birds' habitat consisted of upper subalpine coniferous forest patches that were not regularly visited by humans despite the vicinity of ski tracks. The radiomonitored birds showed a classical bimodal activity pattern with two periods of feeding, 1-2 h each, at dusk and dawn, resting inside snow burrows for the rest of the day[22,23]. Black grouse always burrowed a new igloo after a feeding session, at least as long as snow conditions were appropriate. The experiment consisted of flushing the birds from their snow igloos on 4 consecutive days between 14:00 and 15:00 and collecting all freshly defecated droppings from individually located igloos. The faecal material from one igloo consisted of 4-36 droppings (n = 182 droppings in total) that were deposited between the return from the early morning feeding and the time of flushing, over a period of 7-10 h in a row, but within 18-24 h after a previous experimental flushing. Faeces collected on the first initial flushing event represented our control as they were excreted prior to any experimental disturbance (i.e. flushing); the three subsequent days were our treatment. The droppings were transported in liquid nitrogen and then stored in a deep freezer at –22ºC until analyses. The experiment was conducted in February and March 2003.

Comparative analysis

Faecal samples (n = 132) for the comparison of FCM in habitats with various degrees of human disturbance were collected in January-March 2004 from 32 sites [on average, 4.1 ± 0.2 (SE) igloo samples per site] spread across the SW Swiss Alps, over an area of ca 5'000 km^2. The average (± SE) distance among collecting sites was 31.9 ± 0.8 km. Levels of disturbance by skiers and snowboarders were estimated, within 500 m radius around each sampled Black grouse igloo, by counting the number of ski and snowboard traces from aerial photographs taken from an airplane during a single day flight, four days after a

snow fall in March 2004. We distinguished three levels of disturbance by snow sportspersons: 1) zero or minimum (0-1 ski trace; 7 sites); moderate (2-11 traces; 13 sites); high (> 11 traces, 12 sites). This defined a gradient ranging roughly from natural habitats (category 1) to intensive ski resorts areas (category 3).

Laboratory analyses of corticosterone metabolites

Faecal corticosterone metabolites (FCM) were quantified by a cortisone enzyme immunoassay (EIA)[2], which was first established to measure glucocorticoid metabolites in chickens[24], and then validated with Black grouse[7]. In short, droppings were desiccated (3 h at 70°C), homogenized, and 0.5 g random aliquots were extracted in 5 ml of 60% methanol. These aliquots were diluted 1:10 in assay buffer (pH 7.5) and submitted to the EIA procedure[2]. For the flushing experiment, each dropping was analysed separately in order to collect information about the within-sample variation (i.e. among faecal pellets collected from one igloo). In the comparative approach among habitats with various degrees of disturbance, 4-10 random subsamples per igloo sample were analysed, and we used the sample median for subsequent statistical treatment. In the latter analysis, interassay coefficient of variation was 11%.

Statistical analysis

We used linear mixed models for the analyses of both data sets. Because of unequal sample sizes among categories we did not use standard maximum likelihood estimations, but the method of residual maximum likelihood estimation (REML)[25]. In the field experiment, we considered the factor individual and the interaction of individual and day (categorical) as random terms. The fixed model included the factor day (continuous) as well as its quadratic term. The latter was used to test for a possible curvilinear increase of FCM during the course of the experiment. The residuals of the overall model were normally distributed (Anderson-Darling test, $A^2 = 0.48$, $p > 0.15$). The comparative study required a nested design analysis, with the factor disturbance level (treatment) as fixed term and the collection site, nested within disturbance level, as random term. Faecal samples (igloos) were the residuals. The residuals of the overall model were normally distributed (Anderson-Darling test, $A^2 = 0.44$, $p > 0.15$). All statistical analyses were performed with GENSTAT 5.41[25].

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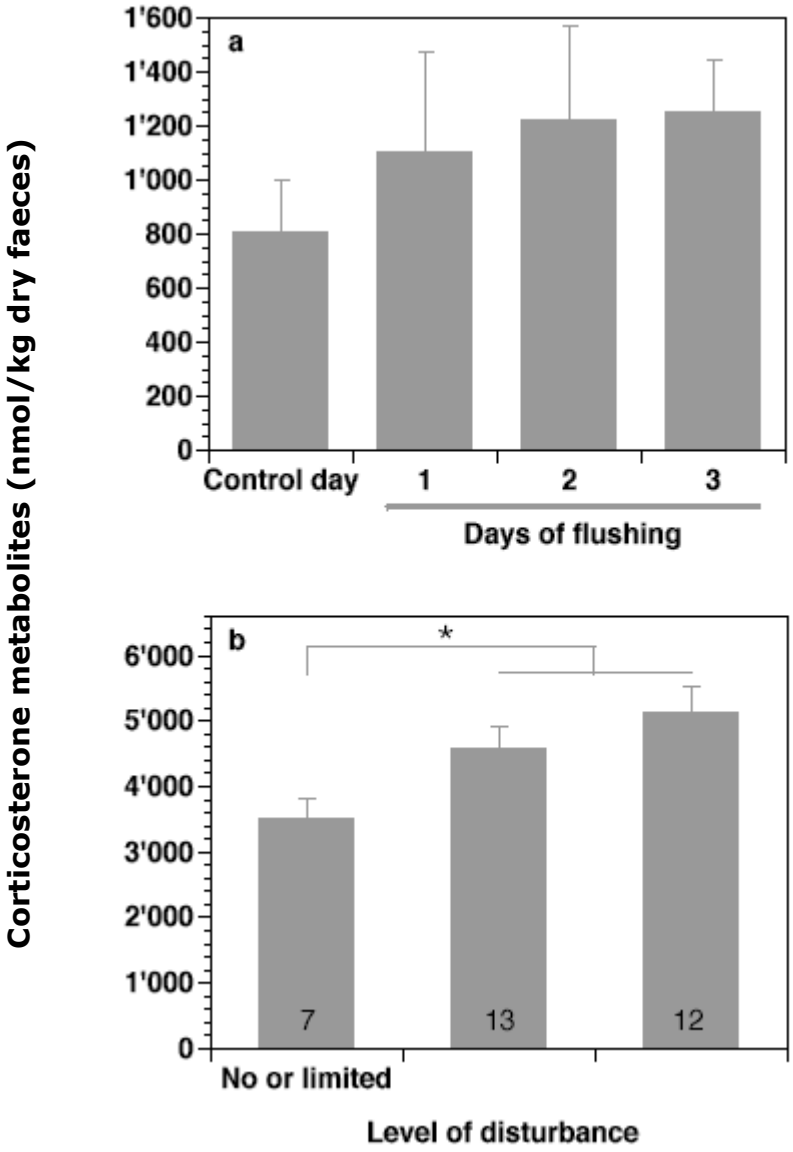
Figure legend

Figure 1. Stress level faced by wild free-ranging Black grouse, measured as the concentration of faecal corticosterone metabolites (FCM, mean ± SE, in nmol/kg of dry material) in droppings collected from individual snow burrows. **a**, Results of a disturbance experiment with three radiomonitored males that were experimentally flushed in winter, once a day over four successive days. FCM levels increased significantly from control day throughout to day 3 of experiment (linear mixed model); **b**, FCM concentrations in the droppings collected from 32 sites (totalling 132 igloo samples) with various levels of disturbance by skiers and snowboarders (sample size at column foot). FCM concentrations differed significantly between sites with no or limited human disturbance vs sites with either moderate or high disturbance; the latter two categories did not differ between each other. * $P < 0.05$; ** $P < 0.01$.



Baltic et al. Fig.1.

Chapter Three

Impact of human disturbance on wintering alpine wildlife: stress, activity budget and energetics in the endangered Black grouse (*Tetrao tetrix*)

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Manuscript

Impact of human disturbance on wintering alpine wildlife: stress, activity budget and energetics in the endangered Black grouse (*Tetrao tetrix*)

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Abstract

1. Human disturbance upon widlife is of growing conservation concern. There are few studies that have looked simultaneously at the impact of man on wild, free-ranging animals from different perspectives, i.e. using different approaches. We investigated stress, behavioural and energetic responses in free-living Black grouse, a declining emblematic species of Alpine ecosystems.
2. Radiomonitored birds were experimentally flushed from their snow burrows in the afternoon, and response to disturbance was quantified from a hormonal, behavioural and energetic viewpoint. Stress hormone metabolites (FCM) were quantified from droppings collected from igloos, as far as possible daily. Changes in activity budgets were reconstitued from the signals emitted by the activity-sensitive radiotags. Energy budgets were modelled to account for possible extra energetic costs induced by disturbance, by linking energy expenditures (data obtained from captive birds in the respirometric laboratory) with *in situ* collected temperature data and activity budgets.
3. There was no significant increase of stress between control and treatment when all nine experimental birds were considered, but the difference showed significantly higher FCM concentrations for treatment (i.e. after artificially elicited disturbance) when a peculiar bird with the highest initial value and a comparatively low treatment value was removed. Overall, initial stress levels showed a large interindividual variation.
4. Birds significantly prolonged foraging duration in the mornings following disturbance by, on average, 23%. In contrast, foraging duration in the afternoon did not differ between treatment and control situations. Disturbed birds were thus potentially exposed to a 12% greater risk of predation by diurnal raptors and carnivores as they did no longer benefit from the protective shelter of igloos.
5. Prolonged foraging time in the mornings following disturbance entailed extra foraging costs that amounted to about 6-9% of overall morning (00:00 to 12:00) energy expenditures (increasing with decreasing ambient temperature), or ca 1.8–3.7% of a whole daily energy budget. These figures represent very conservative minimal estimates as not all components of daily energy budgets could be accounted for, e.g. the costs of sudden escape flights, or the costs of mounting a stress response.
6. *Synthesis and applications.* Winter human disturbance, such as off-piste

skiing or snowboarding, elicits stress and behavioural compensation, which might be detrimental to wintering Black grouse. This study is a new step towards defining tolerance thresholds with regards to winter human disturbance in a vunerable wildlife species of Alpine ecosystems. Implementing conservation measures with the aim to decrease human impact below acceptable disturbance levels for Black grouse will eventually benefit the entire sympatric biocenose.

Keywords: Alpine ecosystems, Corticosterone metabolites, Energy allocation modeling, Foraging activity, Human-generated disturbance, Non-invasive stress monitoring, *Tetrao tetrix*, Tourism

Introduction

The rapidly rising touristical industry increasingly advertises for «active holidays» and «ecotourism» in wilderness. It is largely supported by the development of modern technological and outdoor equipment, which is backed by an aggressive marketing policy for these trendy leisures. Whilst they had up to now remained rather marginal, fun and extreme sports, as well as ecotourism, start to attract a broader and broader public; they are thus progressively becoming an essential financial resource worldwide, with their share of the overall touristical economy growing constantly. On the other hand, these activities have recently been recognized as novel serious sources of threat from the viewpoint of ecosystem and fauna preservation (Fowler 1999; Müllner et al. 2003; Taylor and Knight 2003; McClung et al. 2004). Endangered, declining wildlife which has already to cope with general habitat degradation, due for instance to land use alteration and/or changes in habitat management practices, faces now new additional threats which may affect individual's fitness and population dynamics (Hofer and East 1998; Storch 2000; Ingold 2004). Although there is growing evidence that stress induced by human disturbance is potentially detrimental, there are few studies on free-ranging, wild animal species that have looked simultaneously at all possible repercussions generated by human disturbance, from mounting a stress response, through reallocating time and energy, to individual's physiological state and survival. Optimally, however, all these aspects should be known until adequate conservation strategies can be implemented. In particular, acceptable tolerance thresholds towards human disturbance should be defined. This urges for integrated studies of the actual impact elicited by direct human disturbance upon wildlife. As many wildlife species are currently declining at an unprecedented rate, especially elements of the «mesofauna», this calls for deeper investigations of umbrella species within ecosystems which are more and more suffering from direct human disturbance. By that means, it might be possible to effectively apply corrective measures that may eventually benefit an entire biocenose whose ecological requirements are encompassed within those of the umbrella species (Simberloff 1998).

That human disturbance elicits stress in wildlife is now documented through several studies based on different approaches (Gutzwiller et al. 1998;

Carney and Sydeman, 1999; Fowler 1999; Millspaugh et al. 2001; Taylor and Knight 2003; Blom et al. 2004; Tempel and Gutiérrez 2004). Mounting a stress response is an energetically highly demanding physiological process for the organism (Hofer and East 1998; Svensson et al. 1998; von der Ohe and Servheen 2002; Schummer and Eddleman 2003). The ability of an individual to cope with stress strongly depends on several physiological factors, such as body condition, age, reproductive status, gender (Wingfield et al. 1995; Wasser et al. 1997; Goymann et al. 2002; Touma et al. 2003, Millspaugh and Washbourn, 2004), and even habituation to predictable or unpredictable stressors (Wingfield et al. 1997; Fowler 1999). Yet, little is known about the ways animals trade off stress against other essential components of their life histories, e.g. metabolic maintenance and reproductive aptitude. One of the most crucial determinants of stress appears to be the possibility and capability to quickly restore energetic reserves after a given stressing event. This may be achieved firstly by increasing foraging rate or prolonging foraging bouts *in situ* (Bélanger and Bédard 1990; Brodin 2001; Reneerkens et al. 2002), or by moving to richer foraging grounds (Wingfield et al. 1997). Secondly, artificial prolongation of resting, while lowering metabolic rate, has also been observed (Wingfield and Silverin 1986). Thirdly, mobilizing one's own energetic reserves (e.g. fat deposits, muscle proteins) is another option (Schmidt-Nielsen 1990; Brodin 2001). In any case, an incapability to restore homeostasis leads inevitably towards a physiological weakening of the organism, starvation, easy susceptibility to predators and pathogens and, finally, death (Möstl & Palme 2002; Watson & Moss 2004). This problem would be more acute during winter, when food supply is short and ambient temperature low, and especially among those species inhabiting boreal and alpine ecosystems, which do not store fat and/or are unable to rapidly metabolise proteins from muscle tissues. These animals require constant food supplies (Willebrand and Marcström 1989). For such naturally vulnerable species that have evolved a finely tuned winter energetic physiology, even slight modifications in behaviour and activity patterns may bear enormous detrimental eco-physiological costs (Reimers et al. 2003). In the extreme, stress reaction can be totally suppressed at some critical stages of the biological cycle, e.g. during incubation; this has been observed among some arctic birds inhabiting inhospitable environments, which have to keep energy expenditures to a strict minimum (Wingfield et al. 1995, 1997; Fowler 1999). The aim of this study was too look simultaneously at

several consequences of human disturbance on wintering wildlife, namely stress, activity patterns, time allocation and energetics.

Our study model was the Black grouse (*Tetrao tetrix* L.), an emblematic, umbrella species of Palaearctic, alpine and boreal ecosystems which faces numerous threats at present; this includes severe local population declines and progressive amputation of its European distribution range (Storch 2000). In the European Alps, Black grouse habitat lies around the timberline zone, between 1800 and 2300 m altitude, where most skiing infrastructures are present, rendering it particularly exposed to the development of winter sports. The installation of infrastructures in core Black grouse habitats has been blamed to induce direct additive mortality (Bevanger 1995; Baines & Summers 1997), but more furtive indirect effects, induced by a massive flux of human visitors, are to be expected; those have up to now not been properly quantified.

In winter, Alpine Black grouse shows a bimodal daily activity rhythm, with two periods of foraging close to roosting sites, in early morning and late afternoon (Marjakangas 1986). These two phases usually last 2-3 h, although they may be shorter under severe weather conditions (Pauli 1974; Bossert 1980). The rest of the time, i.e. up to 21 h a day or even more, is devoted to resting, mostly in igloos if snow circumstances allow; snow burrowing has been shown to be an efficient anti-predator and energy-saving strategy (Marjakangas 1986; Spidsø et al. 1997). When snow burrowing occurs, digestion takes place and faecal material is excreted in igloos. Black grouse always dig a new igloo after each foraging bout. This habit of snow burrowing twice a day allows first to potentially collect dated dropping samples, and, secondly, to obtain faecal material stored in optimal conditions for a non-invasive quantification of stress hormone metabolites (freezing temperatures, i.e. no bacterial or enzymatic degradation; absence of sun radiation; Baltic et al., in press). All these characteristics make the Black grouse an excellent model for studying the actual impact of humans on wildlife in alpine ecosystems during winter.

We predicted, firstly, that disturbance elicited by humans actually provokes an increase of baseline levels of stress hormone metabolites (glucocorticoids) in wild, free-ranging Black grouse. Secondly, we hypothesized that the same birds would react by changing activity pattern after reiterated disturbances, namely by prolonging foraging bouts, since Black grouse do not store fat (Willebrand & Marcström 1989). Thirdly, we attempted to model how

behavioural adjustments, if inducing extra energetic expenditures, would affect bird's finely tuned eco-physiological balance in winter.

Materials and methods

Study areas and experimental disturbance

Black grouse males were mist-netted on leks in May 2002 and May 2003 in Verbier (46º2'N, 7º21'E) and Les Diablerets (46º18'N, 7º12'E), two major winter sport resorts in the southwestern Swiss Alps. Nine birds were tagged with 16 g neck-laced radio transmitters equipped with an activity sensor (Holohil Systems Ltd, Carp, Canada), under license of the Wildlife and Game services of the Cantons of Valais and Vaud. Habitat around six birds was irregularly visited by free-riding skiers despite the vicinity of ski resorts, whereas the other three birds stayed among ski pistes, i.e. their habitat was regularly crossed by skiers. Unfortunately, we could not study Black grouse in remote areas with nil or only sporadic human passing. In the study area, most such habitats are in rough terrain that is not accessible in winter (avalanche hazard) and therefore lack necessary facilities for logistics and electronic equipment. In order to minimize the effect of confounding factors (gender, physiological status, etc.), we used only males and ran our experiments from January through to March (2003 and 2004), i.e. prior to the mating period. We attempted to flush each radio-tagged bird from its snow burrow once a day, over 5 consecutive days, between 2 PM and 3 PM. The day before the first flushing event was our control, whereas the four following days (experiment) were our treatment. Unfortunately, not all birds could be experimentally flushed every day during the control and treatment phases: their location was sometimes in inaccessible terrain with furthermore avalanche risk. In our comparison between disturbed vs undisturbed situations, we therefore used, for each bird, the single control value per bird, as well as a single (among days) averaged treatment value, with the two data points obtained per bird eventually constituting a matched pair (disturbance vs non disturbance situations).

Stress response

On each flushing event of a target radiomonitored bird in the afternoon, we

attempted to collect droppings from its snow burrow. Faecal material was excreted during the 6-7 h period prior to flushing, i.e. from the entry into a morning igloo, after dawn foraging, until the experimental disturbance took place. As shown by Baltic et al. (in press, submitted), an entire igloo sample delivered information about the concentration of faecal corticosterone (bird's stress hormone) metabolites (FCM) during a period of up to 18-24 h prior to a flushing event. The droppings were held frozen, first in snow or carbonic ice while in the field, then stored in a deep freezer at –22ºC until laboratory analyses. FCM were quantified by an EIA procedure (Palme and Möstl 1997) with group specific cortisone–EIA (Rettenbacher et al. 2004) that was tested and validated on Black grouse (Baltic et al., in press). In brief, droppings were homogenized and dried until constant weight (3 h at 70ºC). From each igloo sample, 3-10 subsamples (0.5 g each), according to the amount of material collected from an igloo, were randomly selected. FCM were extracted from these subsamples in 60% methanol. Extracts were diluted 1:10 in assay buffer (pH 7.5). Aliquots (10 µl) of standards (4-pregnene-17a, 21-diol-3,11,20-trione; range 2-500 pg/well) and subsamples were incubated overnight, in duplicate, at 4°C, with the label (100 µl, 4-pregnene-17a, 21-diol-3,11,20-trione-CMObiotiniyl-LC; 1:5'000'000) and the group specific antibody cortisone-EIA (4-pregnene-17a, 21-diol-3,11,20-trione-21-HS:BSA; 1:20'000). FCM concentration (in g of dry faeces) of an igloo sample was calculated as the median of all subsamples drawn from that single igloo faecal material.

Activity budget

Thanks to the activity-sensors of the radiotags, birds' activity patterns could be reconstructed from radio signals received from a stationary multidirectional aerial, located on a vantage point overlooking birds' habitat, and recorded with appropriate radioreceiving equipment placed in hermetic boxes burrowed in snow (TR-5, Telonics, Mesa, Arizona). Radiotelemetry of behaviour was achieved constantly during the disturbance experiment period (4 days, hereafter: treatment), as well as on the control day just prior to initiating the experiment (hereafter: control day). Receiver dataloggers registered active signal date and time, pulse period and width (ms) as well as relative signal strength (several thousands records per bird and day). Rapid signal pulse rates corresponded to activity, slow pulse rates to immobile resting. In order to obtain daily activity

patterns, which were comparable among days and birds, activity records had to be standardised (Appendix 2). We first divided days in 10 minutes period intervals, then calculated, for each 10 min period, the absolute number of signals recorded. By dividing those 10 min figures by the total number of data recorded per day, we obtained relative curves of signal pulse rates, i.e. activity curves (Appendix 1). Visual observation of radiotracked birds showed that behaviour could be coded in a dichotomic way since there were two predominant, almost exclusive types of activity in winter: foraging and resting. Time spent grooming or commuting (walking or flying) was a negligible fraction of the total. We thus recoded the activity curves accordingly: were considered as foraging every single 10-min time interval above a 4% threshold of overall daily relative activity, as well as any cluster of three or more 10-min time intervals in a row with values above a 1% threshold. Simple, binary activity budgets could then be drawn (Appendix 2), and duration of morning and evening foraging bouts, as well as resting periods were calculated separately for every bird and day. Note that six birds were included in this analysis given that, due to technical problems, we failed to collect data on control day for 3 cocks.

Energetics

Our ultimate goal was to quantify the real physiological costs endured by the birds in the wild. Yet, metabolic rates with respect to ambient temperatures (T_a) could not be recorded directly in nature, and we had to rely on laboratory respirometric equipment and captive birds instead. Although Rintamäki et al. (1983) already measured energetic consumption in relation to T_a on captive Scandinavian birds, we thought it would be wiser to replicate similar measurements with Alpine birds. Three captive-born Black grouse males, of certified Alpine origin, were bought form Bern Tierpark as chicks and transferred into the spacious aviaries of the Hasli ethological station (Bern University). Birds were kept in captivity under license of the cantonal veterinary service, Bern. They were fed a specific winter grouse diet (Protector SA, CH-1522 Lucens, Switzerland) during the period of experiments.

Respirometric metabolism

Measurements of oxygen consumption (VO_2) took place at the University of Lausanne, Switzerland. During that time, birds were placed individually in a

semi-outdoor enclosure where they stay for a maximum of three consecutive weeks. We technically adapted for Black grouse the method outlined in Arlettaz et al. (2000) and Giorgi et al. (2001). VO_2 was measured at 7 different temperature levels (–20, –10, –5, 0, 5, 10 and 20 ºC) in a plexiglas (1-cm thick walls) metabolic chamber (40 x 40 x 40 cm), which was placed in a thermo-regulated climatizer (Appendix 1). During experiments, birds stayed in total darkness, but their behaviour was monitored continuously using infrared video equipment. In the chamber, they were exposed to a constant airflow at 124 $l*h^{-1}$ down to –5°C. Below –5°C, airflow had to be increased to 149 $l*h^{-1}$, due to a higher metabolic rate, so as to maintain oxygen concentration in the chamber above 20%. Before entering and after exiting the chamber, the air was dried over silica gel; in addition, expired H_2O and CO_2 were absorbed by KOH at chamber exit. Airflow was controlled continuously by a calibrated mass flow meter (5850 E, Brooks Instruments, Veenendaal, Netherlands) connected to a control and read out equipment (5878 E, Brooks Instruments). Oxygen concentration was measured by an oxygen analyser (Gas purity analyser Xentra 4100, Servomex, Esslingen, Switzerland) and data acquisition was performed by the Biobench programme (National Instruments, Austin, USA). Before and after an experimental session (4-5 h per session), the system had to be stabilized for at least one hour to get an exact baseline of oxygen concentration. For each bird and temperature level, we calculated mean VO_2 per time unit and body mass (ml $O_2h^{-1}g^{-1}$), this during a time interval that presented 3 h of stable, uninterrupted respiration patterns. Oxygen consumption was calculated according to Depocas and Hart (1957): $VO_2 = V_2 \times (F_1O_2 - F_2O_2) / (1 - F_1O_2)$, where VO_2 is the flow rate measured after an animal is removed from the chamber, F_1O_2 is the concentration of oxygen at the beginning of experiment, and F_2O_2 is the oxygen concentration in the chamber after the animal was removed. The mean oxygen consumption per time unit and body mass was calculated and transformed into energy expenditure (19.8 kJ l^{-1} O_2; Lindström & Kvist 1995; Svensson et al. 1998).

Ambient, snow and igloo temperature

During and around the period of field disturbance experiments, ambient temperature (T_a, at 2 m above snow surface) and snow temperature (T_s, measured within snow ca 15 cm underneath snow surface, i.e. approximately at

snow burrow depth) were recorded every 10 min (Squirrel Meter, Typ 1006, Eltec, Haslingfeld, Cambridge, UK) in birds' vicinity. In order to account for the temperature differential within igloo due to bird's metabolic heat production, we equipped a tenth cock with a tail-mounted thermo-sensitive tag so as to collect data on the actual temperature within an igloo (T_i, for igloo temperature) when a bird was present. This enabled us to reconstruct the relationships between T_i, T_s and T_a. Signals from the transmitter were converted into temperature data using calibration curve provided by the manufacturer and controlled by ourselves.

Modelling extra energy expenditures induced by human disturbance

Using respirometric metabolism figures obtained from captive resting birds, we attempted to model the overall daily energetic balance of birds subjected to different T_a and T_i levels, for both the disturbed and undisturbed situations. In the model, we derived T_i for a defined air temperature through a two steps procedure: firstly by calculating the function linking T_s with T_a, and, secondly, by linking T_i with T_s. In order to further infer energetic expenditures with respect to activity type, we relied on average foraging and resting bout durations drawn from activity budgets. However, as actual energetic values for foraging were not available, we used resting values instead, but applied a correction factor which considered that costs of foraging were 2.8 higher than resting costs (calculated from equation 2 in Taylor et al. 1982), this irrespective of ambient temperature level for simplification; this corresponded to an estimated realistic average walking distance of 36 m an hour (0.01 $m*s^{-1}$) in a feeding Black grouse. As daily foraging activity was bimodal, with two activity periods in the early morning and in the afternoon that differ in T_a – mornings (AM) being colder than afternoons (PM) – we had to apply another correction factor (linear regressions of AM-T_a and PM-T_a, respectively, vs average daily T_a). This accounted for the contrasted climatic conditions before midday (from midnight to noon) and after midday (from midday to midnight). We were particularly interested to look at the magnitude of the actual additional energetic costs that might be induced by extra (if any) foraging time in disturbed vs undisturbed birds. For that we assumed that birds spent most resting time within igloos, taking profit from the protective temperature buffer, whilst in reality birds may sometimes also have rested on trees. On the basis of field activity budgets and laboratory respirometric data, we built a graphical model of daily energy budgets in relation to ambient

temperature (T_a). The model aimed at visualizing the extra energy expenditure induced by human disturbance.

Statistical analysis

Comparisons of FCM concentrations and activity budgets between disturbed (experimental treatment) vs undisturbed (pre-experiment control) situations were tested using matched-pair tests. Tests were one-tailed since we predicted that disturbance would lead to an increase in both variables. For FCM, the distribution of differences between treatment and control was not normal (Shapiro-Wilk W-test; FCM: W = 0.7714, df = 8, p = 0.0101) and could not be normalized by standard procedures. We therefore applied a non-parametric test (Wilcoxon signed-ranks test). For foraging duration, we used a matched-pairs T-test given that differences between treatment and control were normally distributed, both for morning and afternoon data (Shapiro-Wilk W-test, morning period: W = 0.918, df = 5, p = 0.4803; afternoon period: W = 0.858, df = 5, p = 0.1753). Other statistical treatments included linear and polynomial fits. All statistical analyses were performed with JMP 4.04 (SAS Institute Inc. 1989-2001). Rejection probability levels were set throughout at 5%.

Results

Stress response

FCM concentrations did not differ between disturbed (treatment) and undisturbed (control) situations when all nine birds were considered (Wilcoxon matched-pairs signed-ranks Test, W = 9.500, df = 8, p = 0.301; Fig. 1). Yet, if the peculiar bird with the highest initial (control) FCM concentration and comparatively low treatment FCM value was removed from the analysis, the difference became significant (W = 0.833, df = 7, p = 0.024). Altogether, the variation in FCM concentrations among birds remained huge (range: 439-3070 nmol/kg FCM before the experimental disturbance), with no particular positioning in this respect of the six cocks inhabiting less disturbed areas, compared to the other three cocks that were potentially more disturbed because closer to ski resorts.

Foraging activity

A clear bimodal daily activity pattern was recognized for each individual, with dusk and dawn foraging bouts (Appendix 2). During the control day, before flushing, the mean (± SE) duration of foraging bouts lasted equally in the morning and in the afternoon: 100 ± 11.8 min and 100 ± 12.3 min, respectively (n = 6 birds; Fig. 2). After disturbance, i.e. during treatment, birds significantly increased time devoted to foraging during the morning, by, on average (± SE), 23.3 ± 8.4 min (matched pairs T-test, t = 2.767, df = 5, p = 0.0395). In comparison, afternoon foraging duration was not affected (t = -0.6961, df = 5, p = 0.5174; Fig. 2).

Energetics

Respirometric metabolism

A decrease of ambient temperature (T_a) induced an increase of oxygen consumption in the three laboratory cocks (Fig. 3); this relationship is best described by a polynomial function of second order: $VO_2 = 1000.65 - 19.4*T_a + 0.365T_a^2$ ($R^2 = 0.975$, $p<0.0001$).

Ambient, snow and igloo temperature

The relationship between mean daily snow temperature, at igloo depth (T_s), and mean daily T_a fits a polynomial model (Fig. 4), with a clear buffer effect of the snow below ca –3°C: $T_s = 220.96909 + 0.18513*T_a - 0.01201*(T_a - 278.138)^2$ ($R^2 = 0.7743$, $p < 0.0001$). There was a linear relationship between mean hourly igloo temperature (T_i, estimated from tail-mounted radiotransmitter) and mean hourly T_s, despite the scatter (not shown): $T_i = 6.53745 + 0.64257*T_s$ ($R^2 = 0.1229$, $p = 0.0146$; note that Celsius degrees [°C] were converted into absolute temperatures [K] to avoid negative values when fitting the model). The correction factors applied for modelling separately morning (AM) and afternoon (PM) energetic expenditures were based on the following two regressions: AM-$T_a = -1.08236 + 1.07272\ T_a$ ($R^2 = 0.881$, $p < 0.0001$); PM-$T_a = 0.318 + 0.9188\ T_a$ ($R^2 = 0.9206$, $p<0.0001$).

Modelling energy budgets

Estimated energy expenditures devoted to foraging activity alone were in a range

of 28–42% (70-150 $kJ*kg^{-1}$) of the whole morning (00:00–12:00) energy expenditures; they increased with decreasing T_a from 5°C down to –20°C (Fig. 5). The duration of morning foraging bouts was, on average, increased by 23% after disturbance. This corresponded to an augmentation of 6-9% of energy expenditures for the whole morning (00:00–12:00), and 1.8–3.7% of the overall daily energetic budget, according to T_a (Fig. 6).

Discussion

This work constitutes one of the first attempts to combine simultaneous measurements of stress, activity and energetic budgets for estimating the actual impact of winter human disturbance on a threatened species of Alpine ecosystems.

We could confirm that disturbance by free-riding skiers and snowboarders provokes stress in the Black grouse (Baltic et al, submitted), but the presentresults were less convincing than in a previous field flushing experiment (op. cit.). Altogether, there was a huge variation among individuals as regards the initial (control), baseline concentration of stress hormone metabolites (FCM). This seems to be the rule in investigations of stress response to disturbance in animals (Silverin et al. 1997; Goyman et al. 2002; Touma et al. 2003; Millspaugh and Washburn 2004). When all nine investigated Black grouse cocks were considered, the difference in FCM concentration between the disturbed (experimental treatment) and undisturbed (control) situations was not significant. Yet, it became significant after removing the individual showing the highest initial values; that individual had also a very peculiar response pattern, in comparison with the others: it is the only bird whose stress level dropped dramatically after disturbance (Fig. 1). So, there actually seems to be a «positive» reaction to repeated human disturbance in terms of stress, with an apparent trend in those individuals with high initial FCM concentrations to decrease stress levels after disturbance, whereas individuals with low initial FCM values tended to mount a stress response. Larger sample sizes are now required to see if this might be a common phenomenon. In already highly stressed individuals, it might well be that the only possible strategy to modulate stress response is to suppress it, as observed in arctic birds exposed to severe weather

conditions during breeding (Wingfield et al. 1997). At a certain point, when stress has reached a high level, it might be more beneficial to suppress any further stress response escalation so as to preserve other traded-off vital functions. This solution certainly bears inherent costs, such as release of vigilance. Decrease of vigilance would not be a problem in environments where the sources of disturbance are limited (e.g. the arctic, with sporadic disturbance by predators), but could dangerously expose to great hazards animals inhabiting more speciose communities with larger predator guilds, and/or ecosystems more prone to human disturbance, which is definitely the case of the Black grouse in the Alps. On a broader scale, we have previously shown that basal stress level is higher in Black grouse populations living near Alpine ski resorts or in more natural habitats with frequent human disturbances, than in undisturbed areas (Baltic et al., submitted). Interestingly, the first two categories above (i.e. vicinity of ski resorts, vs areas outside ski resorts with frequent visits by snowboarders and skiers) did not differ between each other in FCM concentrations (Baltic et al, submitted). This might be a first indication that birds in heavily disturbed zones might systematically suppress stress response to ensure the maintenance of all necessary vital functions: their average stress levels would therefore be a secondary, long-term adjustment to repeated perturbations, what we could call a «chronic stress» effect, with its inherent detrimental physiological modifications (Hofer and East 1998). In contrast, birds in less regularly visited and disturbed zones would show FCM levels reflecting reactions to sudden events, e.g. unpredictable flushing by passing-by skiers («sporadic stress»).

Our field experiment showed that repeated disturbance, i.e. daily flushings of the radiomonitored birds in the afternoon, provoked a prolongation of the time devoted to foraging on the following morning. A similar compensatory mechanism has been described earlier in birds and mammals (Gutzwiller et al. 1998; Bélanger and Bédard 1990; Frid and Dill 2002; Fortin and Andruskiw 2003; Reimers et al. 2003, Taylor and Knight 2003). In the Black grouse, compensation took place on the first possible opportunity after stressor's influence was over.

Apparently, birds attempted to replenish energetic reserves almost instantly to avoid short-term alterations of their metabolic balance. This may reflect the fact that Black grouse cannot rely on fat reserves during winter but

have to mobilize muscle proteins (proteins cannot be stored like fat) if they require sudden extra sources of energy (Willebrand and Marcström 1989). We conclude that even when disturbance occurs once a day only, it has direct detrimental consequences on birds' finely tuned winter activity and energy budgets, which urges for rapid energy restoration. In addition to the costs of a sudden escape flight and of mounting a stress response, prolonged exposure to ambient temperatures after disturbance (instead of profiting from the igloo temperature buffer; Marjakangas 1986) is probably the main cause of this need to rapidly reequilibrate the physiological balance. Of course, the problem would become more and more acute with decreasing ambient temperatures, since birds will have to thermoregulate more intensely.

Our try to model extra energetic expenditures generated by winter disturbance was based on estimations of energy consumption obtained from laboratory birds. The figures we obtained differed relatively from the data collected by Rintimäki et al. (1983). The shape of the respective oxygen consumption curves looked similar, without distinct inflection point and no thermoneutrality achieved in the range of temperatures measured. Yet, Scandinavian Black grouse appeared to consume about 50% more oxygen, and their increase in oxygen consumption at lower temperatures was more pronounced. This discrepancy can have several causes. The origin of the birds may be a first explanation: we used Alpine birds whilst Rintimäki et al. (1983) took measurements on birds from Finland (Oulu, 65°N). Weathers (1979) calculated that, in birds, metabolic rate changes, on average, by 1% per degree in latitude. This could explain a substantial proportion (≥20%) of the lower metabolic rate in birds from the Swiss Alps compared to birds from Scandinavia. The remaining variation remains difficult to interpret, however. It may stem from differences in measurement procedures, and/or acclimatization or adaptation of captive birds to cold.

In our energetic model, extra time devoted to foraging after disturbance represented 1.8–3.7% of the overall daily energy use of birds, which is not negligible. Of course, we have to question the validity and limits of our model. Again, our values certainly represent minimal, highly conservative estimates. First, we could not rely on data on exact metabolic costs of foraging and had to extrapolate them from published general equations (Taylor et al. 1982). Instead, we could have used field energetical approaches (the doubly-labelled water

technique for instance; Speakman 1997), but these require recapture of birds every day or every second day, which is almost unfeasible with wild, free-ranging Black grouse in the rough Alpine terrain. Second, emergency escape flights incurred high energetic costs, which could not be appropriately accounted for here. Notwithstanding these limitations and uncertainties in our model, it appears clear that even slight pejorations of the finely-tuned winter energetic balance of Black grouse, particularly if deterioration is repeated, may have tremendous consequences on birds' physiological condition, fitness and, eventually, reproductive aptitude and survival. Finally, we have to stress that one disturbance event a day does not represent a worst-case scenario as flushings may under some circumstances occur even more frequently, especially close to ski resorts. This would further amplify the problems described above.

The extra time spent outside igloos, caused by prolonged morning foraging bouts after disturbance, represented an increase of 12% in daily exposure time to predators. This may be another serious supplementary risk if one considers that predation is the main mortality source in Alpine Black grouse (Rotelli, pers. comm.). Last but not least, frequent disturbances may obstruct access to optimal foraging grounds (West et al. 2002) and induce starvation. In this respect, it has been established that Capercaillies (*Tetrao urogallus*), which are not able to store energy in the form of fat deposits, like Black grouse with which they share numerous life history traits, cannot survive more than nine days of food deprivation. Given that the Capercaillie is the grouse species with the highest capacity to withstand starvation (Hissa et al. 2003), thanks to its huge body size (surface to unit volume ratio), we can expect even a higher susceptibility to starvation in the Black grouse.

Although this study represents one of the first attempts to combine several approaches to quantify the effect of human disturbance on a wild, free-ranging species of Alpine ecosystems, it let numerous questions open despite it could assess alterations in birds' physiological state (stress) and behavioural adaptations. Further investigations are needed; first, to quantify the actual energetic costs of mounting a stress response; second, to estimate the real costs of active vs resting birds; third, to link these physiological, behavioural and energetic impacts with proper survival and reproductive rates. This will demand a huge additional research effort given the difficult working conditions on a rare species inhabiting hostile Alpine ecosystems. Ultimately, however, this would

enable one to define more precisely tolerance thresholds towards human disturbance in winter so that specific, efficient conservation and management schemes can be implemented. As the Black grouse plays the role of an umbrella species for the «timberline biocenose», conservation measures applied for safeguarding it would certainly benefit an entire ecosystem and its wildlife.

Acknowledgements

We thank Prof. Heinz Richner, Bern, who allowed us to use the aviaries of the Hasli ethological station, as well as Olivier Roth who helped with captive birds. Maud Giorgy, Lausanne, gave advices in the respirometry lab. The ski resorts of Verbier and Les Diablerets provided free accommodation and ski-passes. Stéphane Mettaz, Till Berger and Chatrigna Signorell assisted during field work. This research was funded by a grant from the Swiss National Science Foundation to Raphaël Arlettaz (31-67186.01).

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Figure legends

Fig. 1. Mean (± SE) faecal corticosterone metabolites (FCM) concentration in nine radiomonitored Black grouse cocks during control (faecal samples collected from igloos prior to running flushing experiments) and treatment situations. Dark symbols depict birds in the vicinity of ski resorts; light symbols birds in habitats sporadically disturbed by free-riding skiers and snowboarders.

Fig. 2. Mean (± SE) duration of morning and afternoon foraging periods in six Black grouse males before (C: control) and after (Treat: treatment) the experimental disturbance. Matched-pairs T-test, *: $p < 0.05$.

Fig. 3. Oxygen consumption (ml $O_2 h^{-1} g^{-1}$) in three Black grouse males submitted to different ambient temperatures in a respirometric chamber.

Fig. 4. Relationship between snow temperature at igloo depth (T_s) vs ambient temperature (T_a).

Fig. 5. Estimated morning (00:00–12:00) energy expenditures (kJ/kg body mass) in relation to ambient temperatures, showing the energy expenditures during igloo resting (light grey), foraging (dark grey), as well as minimal additional energy expenditures induced by human disturbance once a day (e.g. flushing from skiers, black).

Fig. 6. Relative extra energy expenditure due to human disturbance once a day, in percent of the overall daily energy budget with respect to different ambient temperatures.

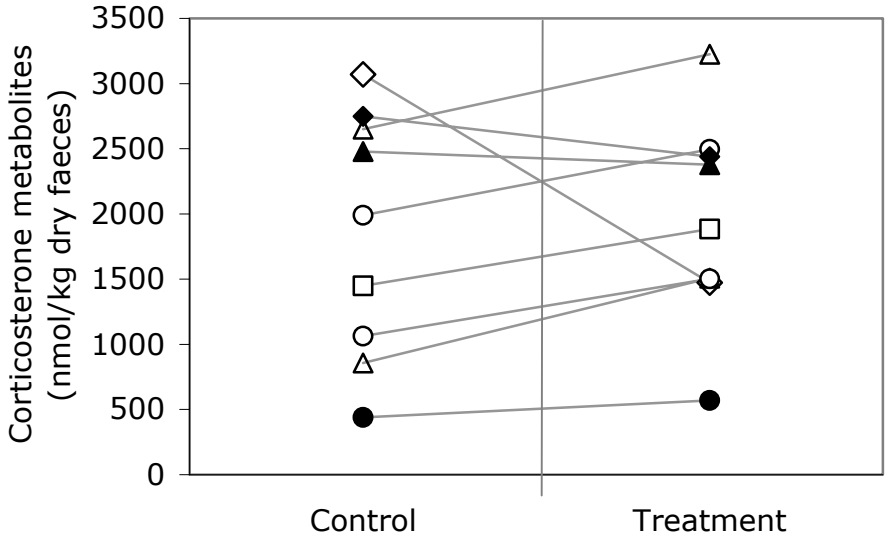
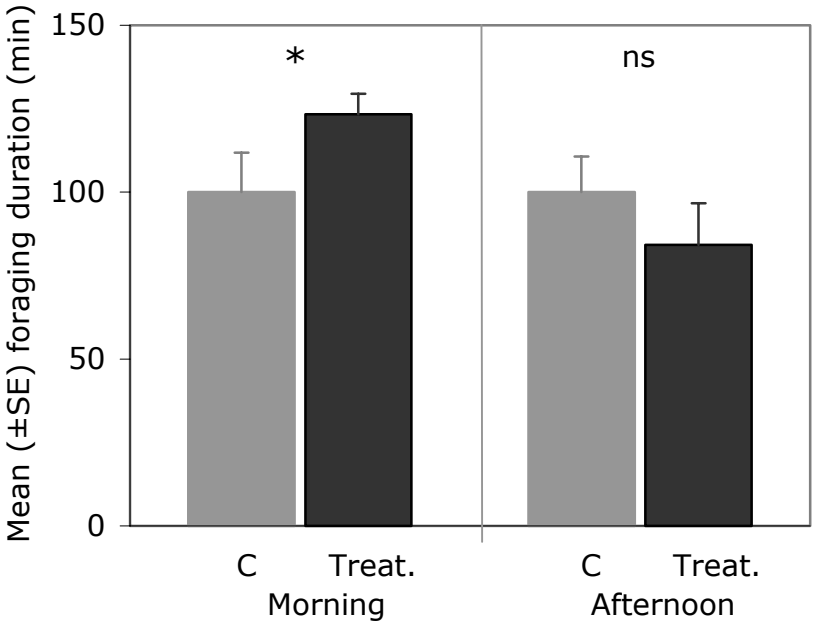


Fig. 1 (Baltic et al.)



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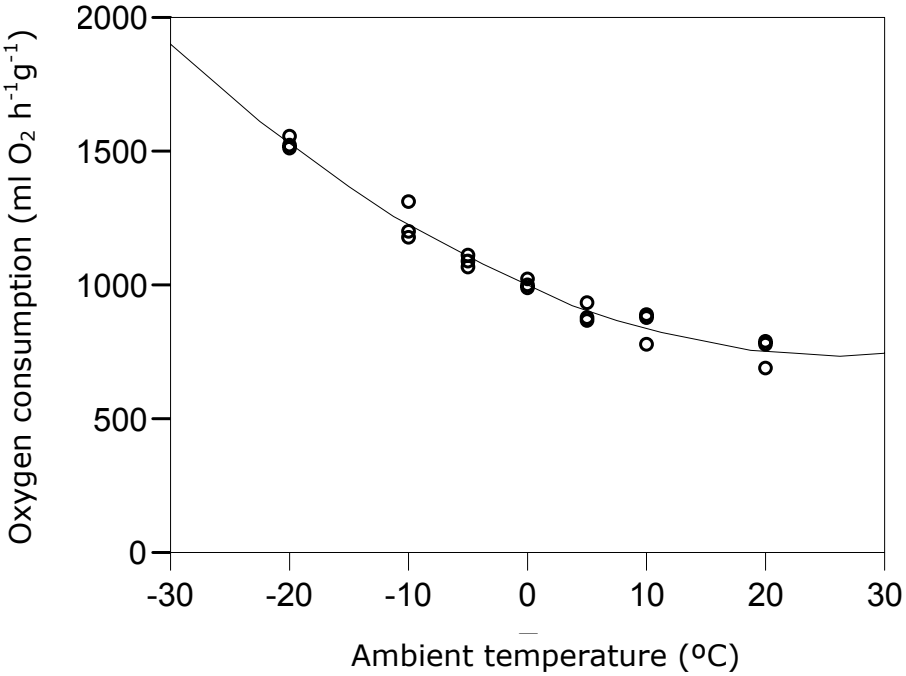


Fig. 3 (Baltic et al.)

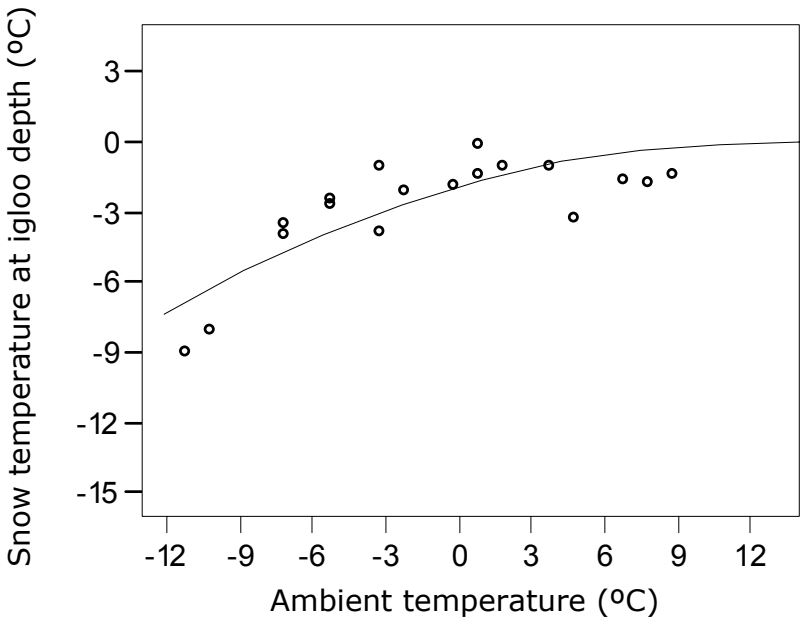


Fig. 4 (Baltic et al.)

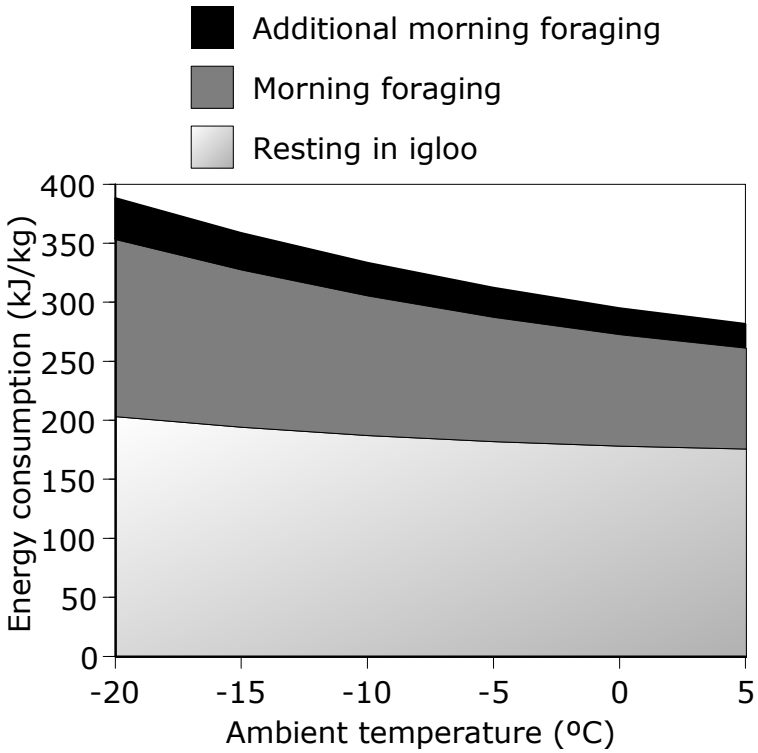


Fig. 5 (Baltic et al.)

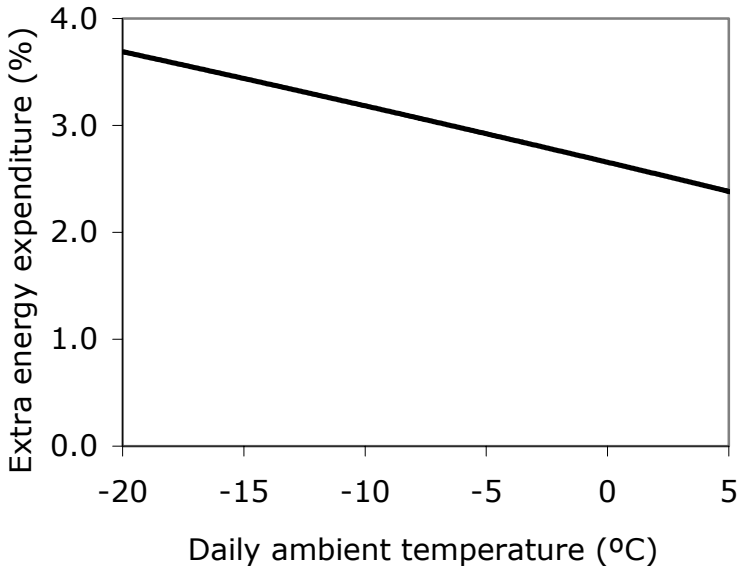
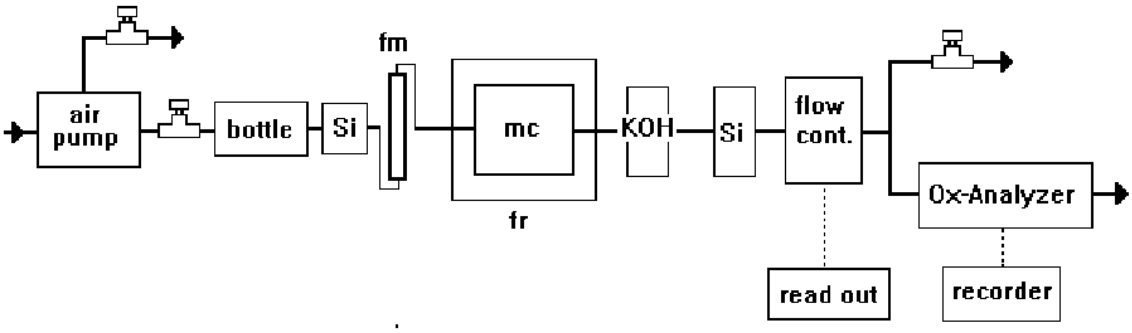


Fig. 6 (Baltic et al.)

Appendix One

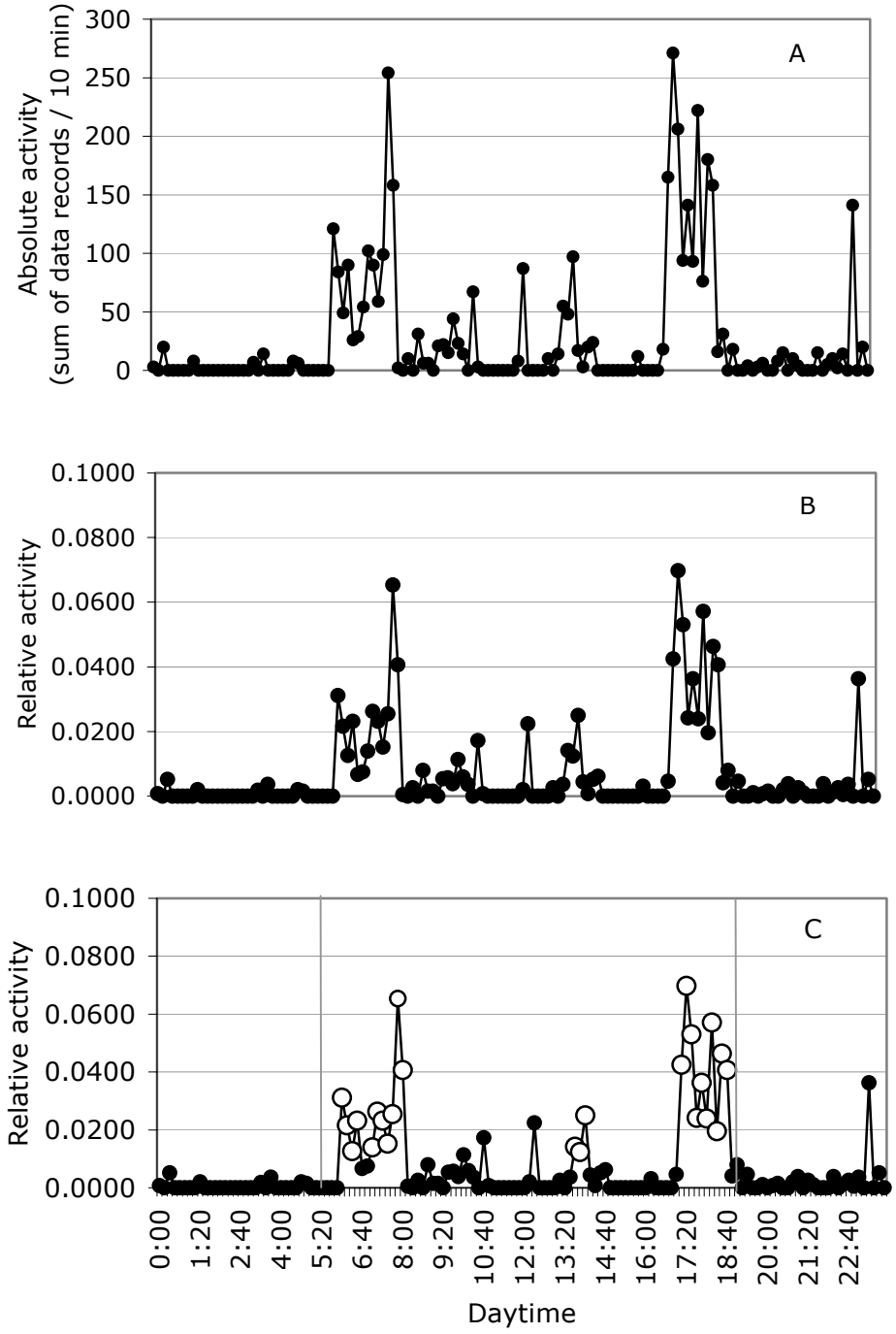
System for measuring respirometric metabolism



System set up for measuring oxygen consumption of the Black grouse in the laboratory. Si = silica gel; fm = flow meter; mc = metabolic chamber; fr = fridge

Appendix Two

Standardization procedures from radio signals towards foraging activity patterns



Appendix 2. Example of an absolute activity curve (A), relative activity proportion curve (B) and activity budget curve (C) for one bird in Les Diablerets on 12th March (144 daily data points, i.e. 10 min periods); white circles represent feeding sequences. The two vertical lines show daylight interval boundaries.

Appendix Three

Curriculum Vitae

Curriculum Vitae

Marjana Baltić

General information:

Born in Zagreb, Croatia, May 19th, 1971

Education:

2001 **PhD Student** at Department of Conservation Biology, University of Bern, Switzerland

2000 **Master of Natural Sciences in Biology (Ecology)** on the Faculty of Mathematics and Natural Sciences of the University of Zagreb, Postgraduate Studies

1995 **Bachelor of Biological Engineering (Ecology)** on the Faculty of Mathematics and Natural Sciences of the University of Zagreb

1990 Mathematical Gymnasium, Zagreb

Project participations:

2001 Croatian Natural History Museum
Position: Research Assistant
Project: "Fauna of the Adriatic Islands of Croatia" with the subject on ecology of insectivorous mammal species

1997 Croatian Natural History Museum
Position: Junior Research Assistant
Projects: 1.) "Fauna of the Adriatic Islands of Croatia" with the subject on ecology of island predators, i.e. Stone marten (*Martes foina*);
2.) "Research and conservation of wetland biotopes and biodiversity of Turopolje, Croatia", with the subject on population dynamics of small mammals

Conference participations:

1994 Second European Congress of Mammalogy in Southampton, England
1996 International Conference on Dormice, Moscenicka Draga, Croatia
1997 19th Mustelid colloquium, Austria
2002 4th International Symposium on Physiology, Behaviour and Conservation of Wildlife, Berlin, Germany
2004 5th International Symposium on Physiology, Behaviour and Conservation of Wildlife, Berlin, Germany

Awards:

1994 Rector's Award of the University of Zagreb for the paper "Fauna, ecology and zoogeography of the butterflies (Insecta, Lepidoptera, Rhopalocera) on Velebit Mts."