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Original Article

Effects of human activity on physiological and behavioral responses of an endangered steppe bird

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Animals may perceive humans as a form of predatory threat, a disturbance, triggering behavioral changes together with the activation of physiological stress responses. These adaptive responses may allow individuals to cope with stressful stimuli, but a repeated or long-term exposure to disturbances may have detrimental individual- and population-level effects. We studied the effects of human activities, particularly hunting, on the behavior and physiological status of a near-threatened nongame steppe bird, the little bustard. Using a semiexperimental approach, we compared before, during, and after weekends: 1) the type and intensity of human activities and 2) the behavior and 3) physiological stress (fecal corticosterone metabolites) of wintering birds. Higher rates of human activity, in particular those related to hunting, occurred during weekends and caused indirect disturbance effects on birds. Little bustards spent more time vigilant and flying during weekends, and more time foraging in the mornings after weekend, possibly to compensate for increased energy expenditure during weekends. We also found increased physiological stress levels during weekends, as shown by higher fecal glucocorticoid metabolite concentrations. Increased corticosterone metabolite levels were associated with the highest levels of hunting-related disturbances. Little bustard showed marked behavioral and physiological (stress hormones) responses to human activities that peaked during weekends, in particular hunting. The long-term effect of this particular activity carried out during weekends from autumn throughout winter might adversely impact wintering populations of this nongame endangered species, potentially counteracting conservation efforts conducted on local as well as foreign breeding populations.

Key words: fecal corticosterone metabolites, flocking behavior, foraging, hunting, *Tetrax tetrax*, vigilance.

INTRODUCTION

Wildlife may perceive humans as potential predators and elicit different antipredator responses (e.g., Beale and Monaghan 2004). Exposure to human activities can modify behavior, physiological status, and ultimately affect the fitness of disturbed animals (Arlettaz et al. 2007; Barja et al. 2007; Ellenberg et al. 2007). In fact, the recently rising intensity and variety of human leisure activities could increase the encounter rate between humans and wild animals and, therefore, their potential negative impact on wildlife (Steven et al. 2011). Among these leisure activities, hunting has received particular attention not only due to its consumptive

nature on game species but also due to indirect effects on their behavior and distribution (e.g., Fox and Madsen 1997; Thiel et al. 2007). However, impacts of hunting on nongame species, including species of conservation concern, are still poorly known (Casas et al. 2009; Sastre et al. 2009).

The effect of human disturbances on animals may be addressed through their influence on spatial distribution, habitat use, or behavioral patterns (Watson and Moss 2004; Sastre et al. 2009). Disturbed individuals may relocate to disturbance-free areas that may be of lower quality (Thiel et al. 2008) or spend more time vigilant to the detriment of foraging or resting (Casas et al. 2009; Wang et al. 2011). Human disturbances may also impoverish individual condition or physiological status (Strasser and Heath 2013; Rehnus et al. 2014), reduce parental care or even prompt nest

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abandonment in birds (Arroyo and Razin 2006; Strasser and Heath 2013).

At the physiological level, organisms exposed to human disturbances may increase glucocorticoid production through the activation of stress responses (Axelrod and Reisine 1984; Zhang et al. 2011; Strasser and Heath 2013). The short-term release of glucocorticoids is an adaptive response that redirects energy from nonvital activities toward survival (Sapolsky et al. 2000). However, the prolonged exposure to stressors and the cumulative effects of maintaining elevated glucocorticoid levels may induce deleterious effects (Romero et al. 2009), such as the suppression of the immune or reproductive functions (Wingfield et al. 1997; Sapolsky et al. 2000). Glucocorticoid levels have been widely used as a physiological indicator of stress in wildlife (e.g., Navarro-Castilla et al. 2014). Fecal glucocorticoid metabolites reflect free glucocorticoids in plasma, yielding an accurate profile of the adrenocortical activity (Sheriff et al. 2010), and offer the advantage of being a noninvasive method for studying wild animal stress responses (Millsbaugh and Washburn 2004). In addition, disturbed organisms may also modify their diet or increase their use of energy stores, which may eventually affect their nutrient balance (e.g., altered nitrogen balance and fecal nitrogen contents in herbivores; Blanchard et al. 2003).

Farmland and steppe bird species are currently the most threatened bird group in Europe, with 83% of species having unfavorable status (Burfield 2005). Agricultural habitats are increasingly humanized, both through the intensification of farming activities and through an increased recreational use of these areas. Human activities may thus have an important and increasing effect on these farmland birds (Onrubia and Andrés 2005; Casas et al. 2009; Sastre et al. 2009), but studies of human disturbance in this context are still scarce. Among farmland birds, the little bustard (*Tetrax tetrax*) is a near-threatened medium-sized steppe bird (BirdLife International 2012) whose populations have dramatically declined in most of its Palearctic range (Goriup 1994). Nowadays, the Iberian Peninsula holds more than half of the world breeding population (García de la Morena et al. 2006). During winter, little bustards gather in large flocks formed by males, females, and juveniles from local and other breeding populations (migratory birds breeding in western France spend the winter in central Spain; Villers et al. 2010). Birds remain gregarious until the beginning of the breeding season, when birds return to reproductive areas and males settle on exploded leks (Jiguet et al. 2000). In winter, little bustards are basically herbivorous, feeding mainly on *Leguminosae* and *Cruciferae* (Cramp and Simmons 1980) and show preferences for new fallows and stubbles (Leitão and Costa 2001; Silva et al. 2004).

Wintering little bustards are confronted with various types of potential human disturbances in agricultural habitats, among which one of the most important is hunting. This is a very important socioeconomic activity in rural areas of the Iberian Peninsula, which takes place in autumn and throughout winter in more than 80% of the territory (Casinello 2013). It is frequently concentrated on weekends, together with other leisure activities, like cycling or hiking. Only indirect effects of hunting disturbance on the species are expected because the little bustard (as occurs with most steppe birds) is not a game species, and therefore, it is not hunted, due to its unfavorable conservation status, but it shares habitat with game species, such as the red-legged partridge (*Alectoris rufa*) and the Iberian hare (*Lepus granatensis*).

We report here on little bustard's behavioral and physiological responses to varying human activity levels, which could be important sources of disturbance. We studied temporal variations in

human activity rates as well as temporal changes in little bustard behavior (flocking behavior, time activity budgets), habitat use, and physiological status (stress levels, evaluated through fecal glucocorticoid metabolites). Finally, we evaluated whether the concentration of glucocorticoid metabolites in feces increased with higher intensities of specific human activities. We predicted that during weekends: 1) human disturbance rates would be greatest; 2) little bustard's flocks would be larger to potentially buffer deleterious effects associated with disturbances; 3) birds would spend more time being vigilant, to the detriment of foraging (although such behavior could be modulated by flock size); and 4) birds would show higher stress hormone levels.

METHODS

Study area

This study was carried out in Campo de Calatrava (Ciudad Real, central Spain) within a Special Protection Area (SPA 157, ca. 38°54'N, 3°55'W). The area is flat to slightly undulated (590–685 m a.s.l.) and is primarily used for cultivation of dry cereal and, to a lesser extent, olive groves (*Olea europaea*), leguminous crops (*Vicia* spp. and *Pisum sativum*), and vineyards (*Vitis vinifera*). Cereal is grown in a traditional way creating a mosaic of sown, ploughed, stubble, and fallow fields of different ages (Table 1). The area has been highlighted as a hot spot for steppe birds (Traba et al. 2007) and holds a significant population of breeding (ca. 100) little bustards, which increases during autumn/winter (ca. 1500–2000) with the arrival of wintering birds (Tarjuelo R, Barja I, Morales MB, Traba J, Benítez-López A, Casas F, Arroyo B, Delgado P, Mougeot F, unpublished data). Hunting modalities are driven shooting for red-legged partridges and walked-up hunting with greyhounds for Iberian hares.

Sampling design

Because hunting is only allowed over weekends in the study area, we followed a semixperimental approach that consisted in comparing data before, during, and after weekends. Hereafter, we refer to data collected before, during, and after a given weekend as belonging to a given "Trial" (or replicate). In winter 2010–2011, trials included data collections over 3 days: Friday, Sunday, and Monday. In winter 2011–2012, we conducted additional surveys on Thursday, Saturday, and Tuesday. For analyses, each survey day was assigned to 1 of 3 categories (hereafter "Week Period"): before weekend (Thursday and Friday), during weekend (Saturday and Sunday), or after weekend (Monday and Tuesday).

Table 1

Habitat availability in the study area during winter (adapted from Martín et al. 2010)

Habitat type	Description	Proportion (%)
Cereal	Crops of barley, oats, or wheat	58.62
Stubble	Recently harvested cereal or legume crops	7.86
Fallow	Fallows of one or more years	7.14
Ploughed land	Ploughed fields without vegetation	9.77
Legume	Crops of <i>Vicia</i> spp. or <i>Pisum sativum</i>	1.36
Pasture	Fields of short scrubland or pasturelands	7.36
Olive grove	Olive tree plantation	3.53
Vineyards	Vine plantation	3.08
Others	Urban fields, stream vegetation, fruit tree orchards, and maize	1.29

Human activity rate and little bustard flock surveys

Quantification of human activities and surveys of little bustard flocks were carried out for 11 trials: 9 during winter 2010–2011 (October 2010–January 2011) and 2 during winter 2011–2012 (December 2011 and January 2012).

We conducted car surveys within the study area using predefined road transects (Figure 1). In winter 2010–2011, transects were longer (32.3 km of tracks or small roads) than in winter 2011–2012 (10.6 km, Figure 1). The density of roads and tracks allowed a full coverage of the places most used by little bustards inside the study area, the covered area being more extensive during the first winter than during the second one. During surveys, we drove at low speed (<20 km/h) and stopped every 500 m at suitable viewpoints to scan for human activity and little bustard flocks, which were georeferenced. We also located human activity and bird observations occurring between stops if they were not detected during stops for scans. We mapped all observations and flock movements, making particular efforts to avoid double counting during surveys (we kept for analysis only observations for which we were confident that they were not sampled twice). Transects were conducted not only in the morning (08:00–14:00 h; $n = 36$) but also in the afternoon for some trials (14:00–18:00 h; $n = 12$). We recorded all little bustard flocks ($n = 113$), including those flying, and noted flock size (number of little bustards; 1–1100) and the habitat used (as one of the following categories: stubbles, ploughed fields, old fallows, cereal crops, vineyards, olive groves, pastures, or urban areas) when birds were not in flight. We also considered mixed flocks with the pin-tailed sandgrouse (*Pterocles alchata*), which are frequently observed during winter (Martin et al. 2010). For human disturbances, we recorded in each transect the number of people, dogs, cars, tractors, and

cyclists. We estimated disturbance rates by type as the total number of people, vehicles, or dogs observed divided by transect length (disturbances/km). Additionally, we recorded the number of hunting shots heard during 5 min at 6 fixed hunting shot sampling points established along transects (Figure 1) and estimated shooting rate (shots/min) for each given transect.

Little bustard behavior

We measured little bustard behavior through behavioral scans during the 2 trials conducted in winter 2011–2012, during morning and afternoon observations. We recorded the proportion of observed individuals being vigilant (i.e., with the neck extended in a typical upward posture), foraging, resting, or walking following the instantaneous scan sampling method (Martin and Bateson 1993). We also recorded date, time, and overall flock size for each scan. The car was used as a hide at a minimum distance of 300 m from the flock, and we used binoculars or a telescope for behavioral scanning. When flock size was less than 20 birds ($n = 4$), we recorded the behavior of all individuals in 1 scan. For large flocks (sometimes up to several hundreds of individuals; $n = 23$), several scan samplings (1–7 scans) were conducted on different groups of individuals within the flock, with a 2-min interval between consecutive scans; in each scan, we sampled the behavior of at least 18 individuals (18–50 individuals). We performed a total of 77 scans (18 in the morning and 59 in the afternoon) in 27 flocks, with an average of 31 ± 9 individuals sampled per scan.

Fecal samples collection

Little bustards in the study area usually gather at night in a single large communal mixed-species roost (of up to 1500 individuals with ca. 1000 pin-tailed sandgrouse) in the same large stubble field or nearby. During the 2 trials conducted in winter 2011–2012, we collected little bustard fecal samples from that large roost at 3 periods: before, during, and after the weekend, to estimate fecal glucocorticoid metabolite concentrations.

Fecal sampling was carried out at dawn after the birds had left the roost (between 08:00 and 09:00). Each morning, we randomly collected 20–30 fresh individual little bustard feces (feces were at least 10 m apart, in order to minimize the possibility of sampling twice the same individual—little bustards move very little when roosting except if disturbed during the night). Fresh feces were characterized by a layer of mucus and the absence of dehydration signals. Each fecal sample was individually stored in a labeled plastic bag and kept refrigerated until taken to the lab (less than 1 h after collection) where it remained frozen at $-20\text{ }^{\circ}\text{C}$ until assayed. We sampled little bustard feces on 4 different days during each trial, with a total of 8 collections and 229 samples. The temperatures at night during fecal sampling were low ($2.3 \pm 1.7\text{ }^{\circ}\text{C}$), which should reduce the potential loss of glucocorticoids with increasing time after defecation, which is temperature dependent (Thiel et al. 2005). We also measured the effects of environmental exposure on corticosterone metabolite concentrations in feces and observed that a loss of fecal corticosterone metabolite concentration occurred only at 12 h after first sampling and was proportional to initial concentration levels (see Supplementary Appendix A).

Glucocorticoid metabolites in feces: extraction and enzyme immunoassay

Fecal glucocorticoid metabolites were extracted from fecal samples according to the modified method of Young et al. (2004). Frozen

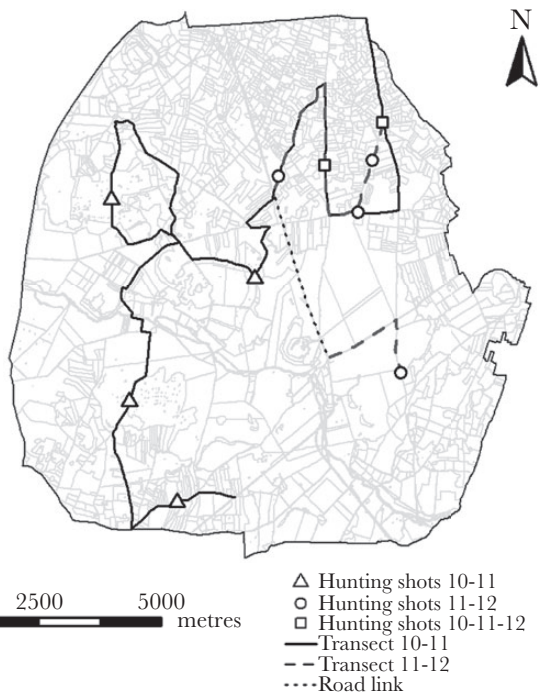


Figure 1

Map of the study area showing the road transects used for surveys during the winters 2010–2011 and 2011–2012. The location of hunting shots sampling points is indicated by triangles (winter 2010–2011), circles (winter 2011–2012), and squares (used both winters).

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AQ4

AQ5

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3.61

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3.110

3.115

3.120

3.122

fecal samples were dried at 90 °C until constant weight, and then 0.08 g of dry samples were placed in assay tubes with 2 mL of phosphate buffer and 2 mL of pure methanol. Tubes were shaken for 16 h and the supernatants were centrifuged at 4000 rpm for 30 min. Pellets were discarded and the fecal extracts were stored at -20 °C until analyzed. A commercial corticosterone enzyme immunoassay (EIA, DRG Instruments GmbH, Marburg, Germany) was used for the quantification. The cross-reactivity of the antibodies with other substances according to the manufacturer was 2.4% for 11-deoxycorticosterone, whereas the cross-reactivity of corticosterone was insignificant (less than 1%) with any other substances (aldosterone, cortisol, and progesterone).

Three tests were done to validate the EIA: parallelism, accuracy, and precision. A parallelism test of serial dilutions of extracts was performed with dilution ratios of 1:32, 1:16, 1:8, 1:4, 1:2, and 1:1, and a curve parallel to those of the standard was obtained. Recovery (accuracy) was $147.4 \pm 36.9\%$ ($n = 6$). Intra- and inter-assay coefficients of variation (precision) were calculated with extracts and the obtained values were $5.3 \pm 1.6\%$ ($n = 6$) and $8.2 \pm 2.8\%$ ($n = 3$), respectively. In each assay, we used a standard (185 ng/mL corticosterone) included in the DRG kit. When standard corticosterone metabolite concentrations deviated more than 10% from the expected value, the assay was rejected and the samples were reanalyzed. The assay sensitivity for corticosterone was found to be > 4.1 ng/mL.

Statistical analysis

We analyzed temporal variations in human activity rates using generalized linear mixed models (GLMMs). Six dependent variables (the number of people, dogs, cars, tractors, cyclist, or shots, counted during a given transect) were fitted to models using a negative binomial error distribution and log link function, with the (log-transformed) transect length (km) included as an offset. For the response variable “number of shots,” we did not include an offset because the number of hunting shot detection points and the time spent there (5 min) were equal for all transects. Variation in little bustard flock size was also analyzed using GLMM with negative binomial error distribution (log link function), considering the (log-transformed) transect length (km) as an offset. Initial models included the following explanatory variables: Winter (to test for possible differences between the winters 2010–2011 and 2011–2012), Week Period (3 levels: before, during, and after weekend), Daytime Period (2 levels: AM vs. PM), and the interaction Week Period \times Daytime Period. Because hunting only occurred during the weekend, GLMMs for the number of shots were built only with the weekend data in order to evaluate differences on shooting rate between winters and daytime periods. The variable “Trial” was included as a random effect (in order to account for the nonindependence of data collected around a given weekend). Nonsignificant ($P > 0.05$) terms were sequentially removed from the initial models, starting with interactions.

For little bustard behavior, we first analyzed factors influencing the probability of whether observed little bustards were in flight or on the ground, using GLMM with binomial error distribution (logit link function). The response variable was a 2-vector function: number of individuals on flight/number of individuals on the ground. Models included Trial as random factor and the same explanatory variables as for flock size models. We analyzed variations in the proportion of individuals foraging or vigilant during scans using generalized linear models (GLMs) with binomial error distribution adjusted for overdispersion and a logit link function. The dependent

variables were 2-vector functions (number of individuals vigilant or foraging/number of individuals sampled during scan performing a different behavior). We used GLM because data were available only for 2 replicates (winter 2011–2012), including Trial as a fixed effect. We also included Week Period, Daytime Period, and Flock size (log-transformed) as well as all possible 2-way interactions between these variables as explanatory variables. We log-transformed flock size because preliminary analyses indicated that the best relationship between behavioral rates and flock size was log-linear rather than linear (with saturated changes for the largest flock sizes). Flock size is known to affect individual behavior, in particular vigilance, which may decrease with increasing group size (Manor and Saltz 2003; Casas et al. 2009). Nonsignificant variables were sequentially removed from the initial full model, starting with interactions.

We performed GLMMs with binomial error distribution for each substrate type to evaluate whether habitat use frequency varied before, during, and after the weekend, using data from both winters (2010–2011 and 2011–2012; $n = 39$). We considered the following substrate types: stubble, cereal, ploughed lands, and old fallows (the other habitats being infrequently and only marginally used). Models included Trial as random factor and Week Period and Daytime Period as explanatory variables. We did not include the interaction Week Period \times Daytime Period due to the sample size.

We analyzed variation in fecal corticosterone metabolites (log-transformed to normalize the variable) using GLM with Gaussian error distribution. We included Trial (2 levels: December 2011 and January 2012) and Week Period (3 levels: before, during, and after weekend) as explanatory variables. Secondly, we explored the associations between little bustard fecal corticosterone metabolite levels and specific human activity rates using also GLMs with Gaussian error distribution. Fecal glucocorticoid concentrations measured in the samples collected at dawn on a day t were indicative of physiological stress levels the previous day (t_{-1}) due to the delay between the stressing events and the increase of corticosterone metabolites in the feces (Nakagawa et al. 2003; Staley et al. 2007). Thus, we used the human activity frequencies observed in the day previous to the feces collection for this analysis. Frequencies of each activity type were categorized into low, medium, and high (see Figure 5 for interval values).

All analyses were conducted using R version 3.1.1 (R Core Team 2014).

RESULTS

Temporal variations in human activity rates

Human activity rates did not differ between winters (2010–2011 and 2011–2012) but strongly differed between week periods (Table 2). More people were observed during weekend mornings (Table 2 and Figure 2). Dogs were more frequently observed during weekends and more abundant in the mornings than in the afternoons (Table 2 and Figure 2). More cars and cyclists were observed during the weekend than before the weekend although no differences were found after weekends (Table 2 and Figure 2). Hunting shots were only reported during weekends and were more frequent during mornings (Table 2 and Figure 2). In contrast, tractors tended to be less frequent during weekend afternoons (marginally significant estimate; Table 2 and Figure 2). Regarding people and dogs observed during weekends, 95% and 99% were hunters and hunting dogs, respectively, whereas on weekdays, observed people were mainly farmers (68% and 77% before and after, respectively),

Table 2
Results of the GLMMs testing for temporal variations in human activity rates, little bustard flock sizes, and flying probability

Response variables ^a	Explanatory variable	χ^2	df	<i>P</i>	Variable level estimates ^b	Estimate	<i>z</i> -Value	<i>P</i>	
5.5 People/km ^c	Week Period	39.80	2	<0.001	Intercept (a)	-0.410 ± 0.299	-1.37	0.170	5.65
					Weekend (b)	2.334 ± 0.372	6.27	<0.001	
	DTP	1.09	1	0.296	After (a)	0.470 ± 0.403	1.17	0.244	
					PM (a)	0.588 ± 0.562	1.04	0.296	
					PM × Weekend (a)	-2.906 ± 0.800	-3.63	<0.001	
5.10 Dogs/km ^c	Week Period	19.06	2	<0.001	PM × After (a)	-0.319 ± 0.778	-0.41	0.682	5.70
					Intercept (a)	-0.858 ± 0.464	-1.85	0.065	
	DTP	7.91	1	0.005	Weekend (b)	2.437 ± 0.605	4.03	<0.001	
					After (a)	-1.125 ± 0.734	-1.53	0.125	
					PM	-2.013 ± 0.716	-2.81	0.005	
5.15 Cars/km ^c	Week Period	14.13	2	<0.001	Intercept (a)	-0.195 ± 0.253	-0.77	0.441	5.75
					Weekend (b)	1.197 ± 0.325	3.68	<0.001	
	DTP	5.97	2	0.05	After (ab)	0.371 ± 0.343	1.08	0.280	
					Intercept (a)	-2.285 ± 0.695	-3.29	0.001	
					Weekend (b)	2.095 ± 0.864	2.43	0.015	
5.20 Tractor/km ^c	Week Period	1.64	2	0.441	After (ab)	0.012 ± 0.972	0.01	0.990	5.80
					Intercept (a)	-0.214 ± 0.295	-0.72	0.469	
	DTP	0.38	1	0.538	Weekend (a)	-0.226 ± 0.229	-0.99	0.323	
					After (a)	0.264 ± 0.236	1.12	0.264	
					PM (a)	-0.281 ± 0.457	-0.62	0.538	
5.25 Shots/min ^c	Week Period × DTP	7.534	2	0.023	PM × Weekend (a)	-1.854 ± 1.097	-1.69	0.091	5.85
					PM × After (a)	-0.578 ± 0.641	-0.90	0.367	
	DTP	5.83	1	0.016	Intercept (a)	3.594 ± 0.441	8.16	<0.001	
					PM (b)	-2.272 ± 0.941	-2.42	0.016	
					Intercept (a)	3.048 ± 0.334	9.14	<0.001	
5.30 Flying probability ^d	Week Period	465.22	2	<0.001	Weekend (ab)	-0.073 ± 0.326	-0.22	0.823	5.90
					After (b)	-0.902 ± 0.335	-2.69	0.007	
	DTP	7.30	2	0.026	Intercept (a)	-5.477 ± 1.141	-4.80	<0.001	
					Weekend (b)	2.030 ± 0.102	19.97	<0.001	
					After (a)	-0.127 ± 0.191	-0.67	0.505	

Overall effects of explanatory variables as well as estimates of each factor level (±SE) and their significance are given.

^aAll models included “Trial” as a random effect. Initial models (Supplementary Table B.1) included the following explanatory variables: Winter (2010–2011 vs. 2011–2012), Week Period (before, during, and after weekend), Daytime Period (DTP = AM vs. PM), and the interaction between Week Period and DTP. GLMMs for number of shots/min used only data from weekend (no shots were recorded before or after weekend).

^bDifferent letters indicate significant differences ($P < 0.05$, Tukey’s HSD) between levels. The intercept corresponds with before weekend and AM levels of the variables Week Period and Daytime Period, respectively.

^cHuman activity variables and flock size were fitted using negative binomial error distribution, with transect length (log-transformed) included as an offset (except for number of shots; see Methods).

^dFor flying probability (i.e., whether an observed flock was in flight or on the ground), we used binomial error distribution and a logit link.

5.40 but never hunters. The number of cars during weekends correlated positively with the number of hunters ($\rho = 0.53$).

Temporal variations in little bustard behavior

5.45 Little bustard flock size did not differ significantly between winters and daytime periods but was significantly influenced by week period, with smaller flocks found after weekends (Figure 3 and Table 2). The proportion of little bustards in flight when first detected was also significantly affected by week period: more birds were observed flying during weekends than before or after weekends (Figure 3 and Table 2).

5.50 The proportion of little bustards being vigilant within scanned groups depended on flock size and week period, with a significant interaction between these variables (Table 3). Before weekends, the proportion of vigilant birds increased with flock size (slope ± standard error [SE]: 0.604 ± 0.245 ; Figure 3). In contrast, during and after weekends, the proportion of vigilant birds decreased with flock size (slopes ± SE: -0.529 ± 0.261 and -1.359 ± 0.282 , during and after weekends, respectively, Figure 3).

5.55 The proportion of little bustards foraging also varied with flock size and week periods, depending on daytime period (Table 3).
 5.60 More birds were foraging in the mornings after weekends and during
 5.61 afternoons before weekends (Table 3 and Figure 3). Moreover,

the proportion of birds foraging increased with flock size, this relationship being stronger during mornings (slope ± SE: 0.228 ± 0.153) than afternoons (slope ± SE: 0.173 ± 0.103).

Temporal variations in habitat use

5.105 During winter, little bustards used mostly stubbles (57.2% of the total birds observed), followed by ploughed fields (16.7%), cereal crops (16.2%), and, to a lesser extent, old fallows (9.2%) and pastures or field margins (0.7%). The proportion of little bustards observed in different habitat types differed between week periods and daytime periods (Table 4). Little bustards used stubbles more frequently during the weekend and on the afternoons and tended to use old fallows more often after the weekend (Table 4). The use of cereals was at its highest before the weekend but reduced after the weekend (Table 4). Ploughed fields were used more often after the weekend and on the afternoons (Table 4).

Human disturbances and fecal corticosterone metabolite levels

5.120 Fecal corticosterone metabolite levels varied between week periods depending on trial (Trial: $\chi^2 = 4.19$, $P = 0.041$; Week period:
 5.122 $\chi^2 = 18.61$, $P < 0.001$; Trial × Week period: $\chi^2 = 14.38$, $P < 0.001$).

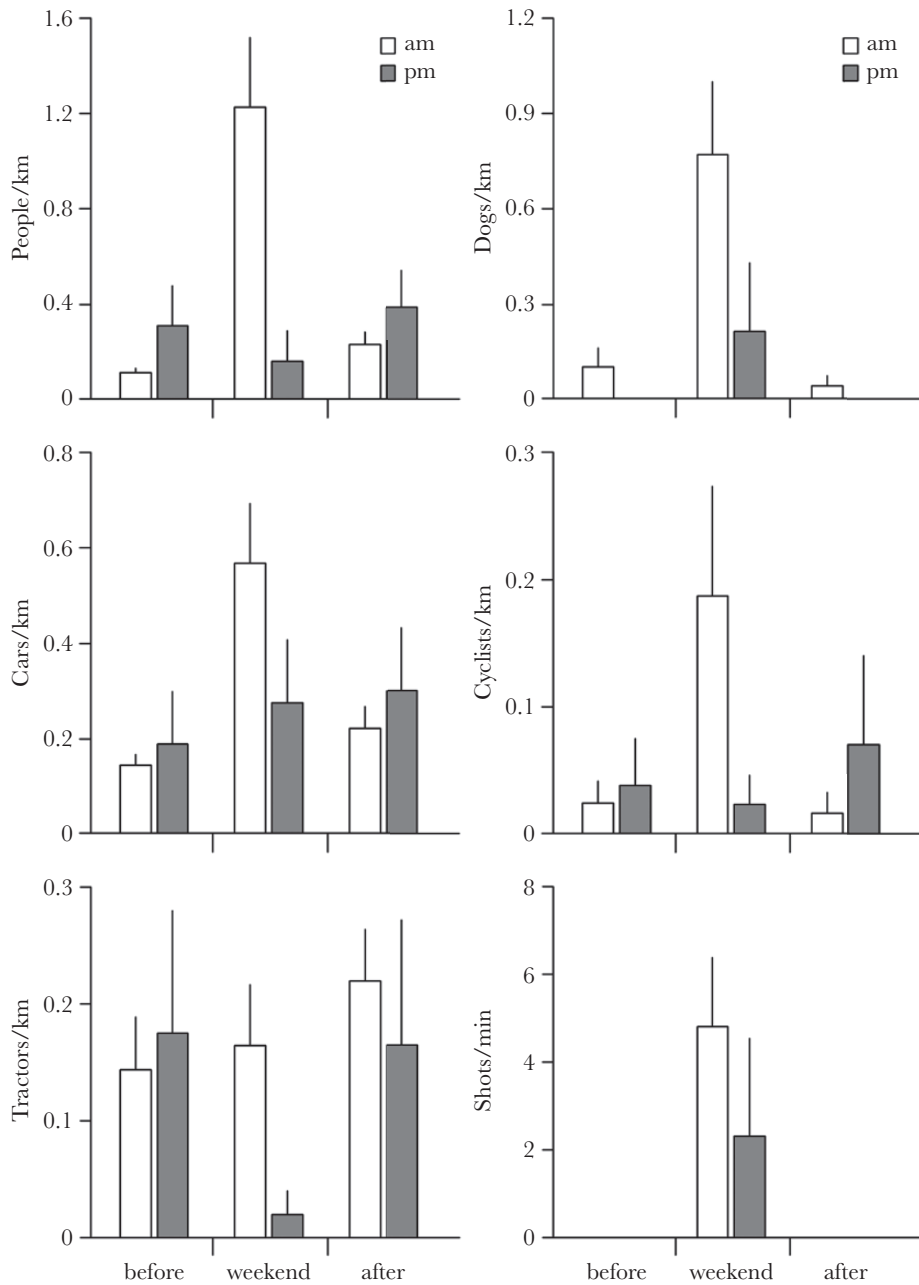


Figure 2
Mean (\pm SE) human activity frequencies according to week and daytime periods.

Fecal corticosterone metabolite levels were greater during weekends than before weekends for both Trials and greater during weekends than after weekends, but only for the January Trial (Figure 4).

We further investigated fecal corticosterone metabolite variations according to specific human activity levels. Corticosterone metabolite levels were significantly affected by the number of people/km ($F_{2,196} = 8.13, P < 0.001$), dogs/km ($F_{2,196} = 7.73, P < 0.001$), cars/km ($F_{2,196} = 8.77, P < 0.001$), and shots/min ($F_{2,196} = 6.41, P = 0.002$), whereas no differences were found for tractors/km ($F_{2,196} = 2.64, P = 0.074$) or cyclists/km ($F_{2,196} = 2.25, P = 0.108$). Post hoc Tukey's HSD tests showed that corticosterone metabolite levels were significantly greatest at both intermediate and high levels of cars/km and shots/min, and they were greatest only for the highest levels of people/km and dogs/km (Figure 5).

DISCUSSION

Our results showed that disturbances due to human activities affected the behavior and physiological stress of wintering little bustards (but see Supplementary Appendix C for nutritional status results). Human presence in our study area during weekdays was overall low and mainly related to farming activities but was much higher during weekends, mainly because of hunting activities. Other leisure activities, such as cycling or hiking, were also more common in the weekends than during weekdays, but with lower absolute frequency than hunting activities.

Disturbances and behavior

Little bustards reacted to human disturbances by modifying their behavior. The probability of detecting birds in flight was higher

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6.95

6.100

6.105

6.110

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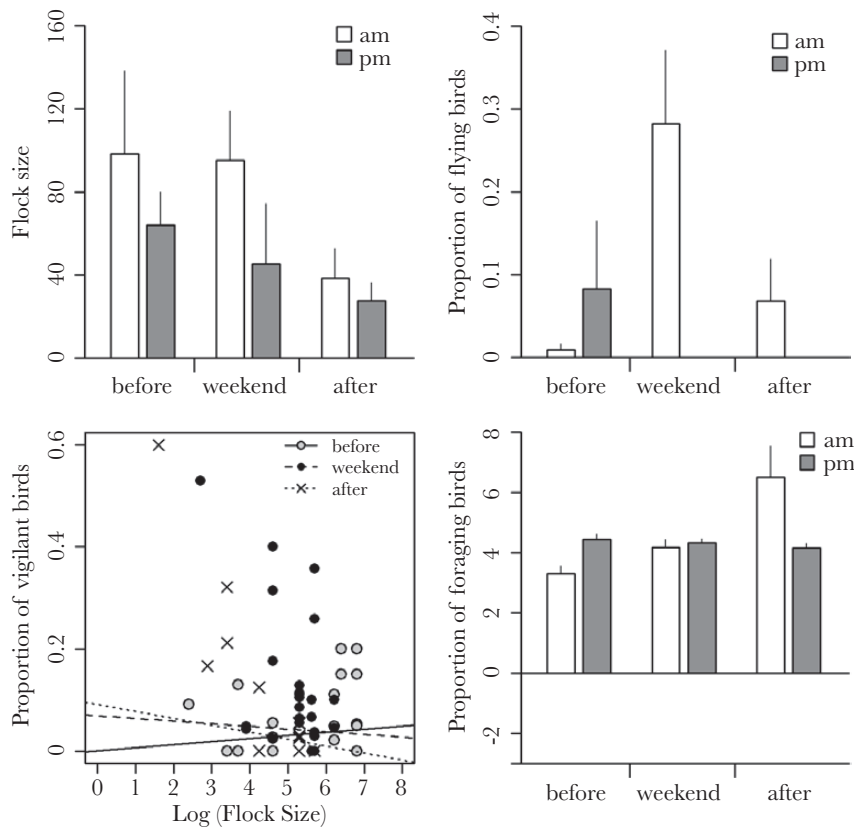


Figure 3 Proportion (mean ± SE) of little bustards that were flying when first detected, flock sizes (mean ± SE), and proportion (least square means ± SE) of little bustards foraging within flocks according to week and daytime periods. The proportion of vigilant birds varied with (log-transformed) flock size, with different slopes for each level of week period (see text).

Table 3 Results of the GLMs analyzing temporal variation in the proportion of little bustards being vigilant or foraging within flocks (n = 77 scans)

Response variables ^a	Explanatory variables	χ^2	df	P	Variable level estimates	Estimate	z-Value	P
Vigilance					Intercept	-6.248 ± 1.629	-3.84	<0.001
	Log(FS)	5.89	1	0.015	Log(FS)	0.604 ± 0.271	2.23	0.029
	Week Period	25.31	2	<0.001	Weekend	6.929 ± 1.964	3.53	<0.001
					After	9.108 ± 2.150	4.24	<0.001
	Log(FS) × Week Period	25.77	2	<0.001	Weekend × log(FS)	-1.133 ± 0.347	-3.26	0.002
Foraging					After × log(FS)	-1.963 ± 0.440	-4.46	<0.001
					Intercept	-3.684 ± 1.173	-3.14	0.003
	Log(FS)	10.92	1	0.001	Log(FS)	0.595 ± 0.196	3.04	0.003
	DTP	8.71	1	0.003	PM	3.546 ± 1.287	2.76	0.008
	Week Period	12.31	2	0.002	Weekend	0.862 ± 0.366	2.36	0.021
					After	3.193 ± 1.094	2.92	0.005
	Week Period × DTP	13.12	2	0.001	PM × Weekend	-0.976 ± 0.426	-2.29	0.025
	Log(FS) × DTP	4.25	1	0.039	PM × After	-3.473 ± 1.115	-3.11	0.003
				Log(FS) × PM	-0.436 ± 0.220	-1.98	0.052	

Overall effects of explanatory variables as well as estimates of each factor level or covariate (±SE) and their significance are given. ^aInitial models included Log(FS) (flock size, log-transformed), Trial (December vs. January), Week Period (before, during, and after weekend), Daytime Period (DTP = AM vs. PM), and all possible 2-way interactions (Supplementary Table B.2) as explanatory variables.

during weekends, indicating that little bustards spend more time flying in response to higher levels of human disturbance. Escape is a crucial response performed by organisms (Ydenberg and Dill 1986) and may be adjusted to perceive risk, increasing the flight initiation distance with risk probability (Frid and Dill 2002; Thiel et al. 2007). The escape movements caused by disturbance could

also lead to the redistribution of individuals toward disturbance-free areas (Casas et al. 2009). Additionally, we found that the relationship between vigilance rate and flock size was influenced by week period. During and after weekends, a lower proportion of birds were vigilant in larger flocks, where more individuals are scanning the surroundings (e.g., Lima 1995) or because

Table 4

Results of the GLMMs (with binomial error distribution) analyzing temporal variation in the proportion of little bustards using a given habitat type ($n = 39$ transects)

Response variables ^a	Explanatory variables	χ^2	df	P	Variable level estimates ^b	Estimate	z -Value	P
Ploughed field	Week Period	666.08	2	<0.001	Intercept (a)	-4.393 ± 1.221	-3.60	<0.001
					Weekend (b)	1.643 ± 0.106	15.51	<0.001
					After (c)	3.168 ± 0.125	25.42	<0.001
Cereal	DTP	44.70	1	<0.001	PM	-1.685 ± 0.252	-6.69	<0.001
					Intercept (a)	-3.390 ± 2.275	-1.49	0.136
					Weekend (b)	-5.041 ± 0.218	-23.11	<0.001
Stubble	Week Period	125.28	2	<0.001	After (ab)	-75.038 ± 48.380	-1.55	0.121
					PM	2.044 ± 0.183	11.19	<0.001
					Intercept (a)	-0.469 ± 0.893	-0.53	0.599
Old fallow	Week Period	353.68	2	<0.001	Weekend (b)	0.874 ± 0.091	9.57	<0.001
					After (c)	-1.135 ± 0.092	-12.36	<0.001
					DTP	1.010 ± 0.111	9.13	<0.001
Old fallow	Week Period	83.36	1	<0.001	Intercept (a)	-5.415 ± 1.438	-3.77	<0.001
					Weekend (a)	0.208 ± 0.133	1.57	0.117
					After (b)	1.553 ± 0.128	12.10	<0.001

Overall effects of explanatory variables as well as estimates of each factor level (\pm SE) and their significance are given.

^aAll models included "Trial" as a random effect. Models included Week Period (before, during, and after weekend) and Daytime Period (DTP = AM vs. PM) as explanatory variables. The model for "Old fallow" did not include the variable DTP (only one observation was recorded during afternoon).

^bDifferent letters indicate significant differences ($P < 0.05$, Tukey's HSD) between levels. The intercept corresponds with before weekend and AM levels of the variables Week Period and Daytime Period, respectively.

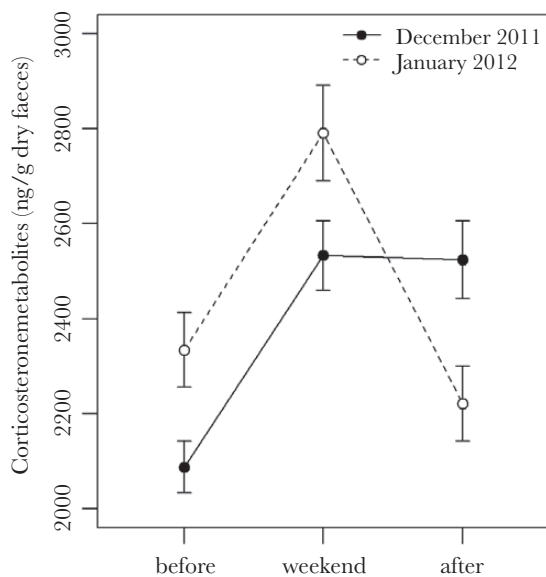


Figure 4
Mean (\pm SE) fecal corticosterone metabolite levels according to week period and trial.

individual predation risk decreases with group size (Hamilton 1971). In our case, the negative relationship between vigilance rate and group size was weaker during weekends than after weekends, suggesting that little bustards exposed to high levels of disturbance exhibit maximum vigilance rates regardless of flock size (Manor and Saltz 2003). Indeed, increased vigilance is an anti-predatory response, which allows individuals to flee from potential predators before an attack occurs (Caro 2005). Our results are in accordance with Casas et al. (2009) who reported higher vigilance rates in little bustards in France during hunting days. In addition, the use of stubbles increased during the weekend, possibly indicating that birds look for safer places, with higher vegetation cover, to spend the night after disturbances.

The different behavioral pattern found between weekends and after weekends might reflect an energy-allocation trade-off between vigilance and foraging activity. The increase in the safety-related responses to disturbance described above could be associated with higher energetic expenditure. The higher energy expenditure and the longer time spent in vigilance during weekends reduced both the energy and time available to other behaviors like foraging. This may also explain the behavioral pattern found in the mornings after weekends, when vigilance rate decreased but when foraging was at its highest, and when little bustards also used old fallows more often, a habitat with potentially greater availability of food resources (Tarjuelo R, Barja I, Morales MB, Traba J, Benítez-López A, Casas F, Arroyo B, Delgado P, Mougeot F, unpublished data). After disturbance events, organisms may need to compensate for the energy losses resorting to increase food intake (Blanc et al. 2006), as observed here. Moreover, little bustard flocks spread in smaller ones after being exposed to higher human pressure, a behavioral response that may improve resource exploitation by avoiding foraging competition (Sansom et al. 2008). Although it has been questioned that reduced vigilance time implies a greater food intake by increasing feeding rate (e.g., Powolny et al. 2012), the change in little bustard foraging patterns after weekends points out to a strategy for recovering energy stores.

Physiological responses to disturbances

Increased human disturbances during weekends were associated with greater glucocorticoid metabolite concentrations in feces. Although physiological stress is an adaptive response to cope with adverse impacts of risky environments and situations, the long-term exposure to elevated glucocorticoids may lead to noxious effects such as physiological damage or immune inhibition, which may in turn affect population growth (Sapolsky et al. 2000; Ellenberg et al. 2007). Importantly, corticosterone metabolites in little bustards remained high after weekends, at least in the December Trial (when shot rate was more intense than in January), suggesting a persistent negative effect that may last at least 1 day after the disturbance events. Fecal glucocorticoid metabolite levels appeared directly

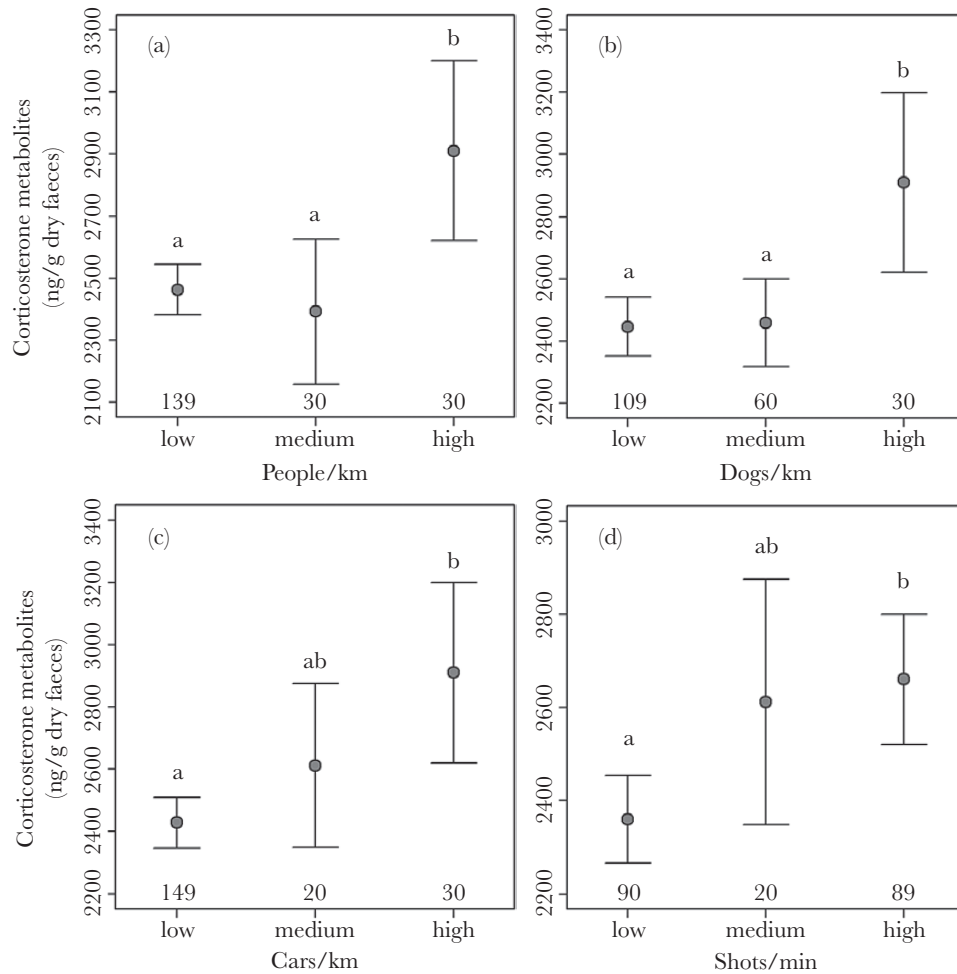


Figure 5

Mean ($\pm 95\%$ confidence intervals) fecal corticosterone metabolite levels according to human disturbance: a) people/km, b) dogs/km, c) cars/km, and d) shots/min. Disturbance frequencies were classified into 3 levels (low, medium, and high). The particular intervals for each disturbance type were 1) people/km: low (0, 1.01], medium (1.01, 2.01], and high (2.01, 3.02]; 2) dogs/km: low (0), medium (0, 1.00], and high (1.00, 2.83]; 3) cars/km: low (0.09, 0.35], medium (0.35, 0.60], and high (0.60, 0.85]; 4) shots/min: low (0), medium (0, 0.50], and high (0.50, 5.20]. Sample sizes for each frequency category are given in the bottom of the graphs. Different letters indicate significant differences ($P < 0.05$, Tukey's HSD) between categories.

associated with certain types and intensities of disturbances, indicating that observed patterns are not merely a temporal association. Little bustard glucocorticoid metabolite levels were particularly affected by hunting shot rates and cars, even at intermediate frequencies, and by high rates of disturbance by people and dogs, which mainly occurred in our study area due to hunting: partridge driven shooting involves large numbers of hunters walking in fields and hare hunting with greyhound dogs involves smaller groups of hunters and dogs covering large areas. For hare hunting, the predatory behavior of greyhounds (running at high speed through fields) could likely mean a higher perceived threat to birds (Lafferty 2001; Martinetto and Cugnasse 2001). The physiological responses of little bustards to this hunting activity could be strengthened by its nonpredictable and intense nature (Beale and Monaghan 2004; Blanc et al. 2006).

By contrast, low or intermediate frequencies of human presence (in our area, associated with individual farmers in fields, or small groups of walkers on tracks), cyclist and tractors working in fields, did not seem to affect little bustard fecal glucocorticoid metabolites. The rather low intensity of cycling in our area, together with the fact that bikers stay on tracks, might explain this lack of effect.

Farmers, tractors, and walkers are more constantly distributed over the week and are present all year round, so they may potentially represent nonlethal predictable events, which may facilitate habituation (Conomy et al. 1998).

In addition to measuring glucocorticoid metabolite concentrations in feces, we also measured the carbon and nitrogen contents in a sample of little bustard feces collected before and during weekend (Supplementary Appendix C). These preliminary data indicate a significant qualitative difference, with carbon content about 10% greater during weekends than prior to weekends and nitrogen content about 1% greater during weekends than prior to weekends (Supplementary Appendix C). These qualitative differences may reflect differences in diet or metabolism (e.g., a greater mobilization of energy stores resulting in increased fecal C content during weekends). The lack of detailed information on the winter diet of little bustards and on the chemical composition of the consumed plant species complicates the interpretation of these data. Future studies could look into more detail at possible changes in diet before, during, and after weekends in order to better link disturbance effects on habitat use, diet quality, and nutritional status.

CONCLUSIONS

Hunting and recreation pressures are increasing worldwide over all areas that are accessible for humans. These areas may still support high-value biodiversity, and understanding the different levels of subtle and direct impacts of such increasing disturbance will be a key to set targeted management strategies for particularly high biodiversity areas (e.g., using time and spatial access restrictions). Our results showed that high levels of human activities during weekends exert a negative effect on little bustards (behavioral and physiological responses consistent with antipredatory strategies; Beale and Monaghan 2004). Hunting appears as a particularly important source of disturbance for this declining species (see also Casas et al. 2009), prompting changes on its behavior and inducing physiological stress, which may even persist after the disturbance has disappeared. Human-induced changes in the behavior and physiology may have consequences on individual fitness (Strasser and Heath 2013), and ultimately on population dynamics (Ellenberg et al. 2007), which could be particularly worrying for this threatened species. The impact of high stress levels associated with hunting activity during winter might have also consequences on the following breeding season. If adults arrive to breeding areas with depleted body condition (induced by maintained high corticosterone levels), they may require longer time to regain the adequate condition to start reproduction. During winter, central Spain hosts not only local populations but also populations breeding in France, which have severely declined and are subject to intense conservation management (Bretagnolle and Inchausti 2005; Villers et al. 2010). Thus, an inadequate management of areas with wintering little bustards may counteract the benefits obtained through conservation efforts focused on breeding populations elsewhere. The creation of hunting-free areas, which harbor good quality foraging habitats for the species, may aid to reduce the impact of hunting activity (Casas et al. 2009).

Further studies should link species physiological, behavioral, and distribution changes to population declines. Because the impacts of disturbances on free-living animals may be detected earlier on individuals than on populations (Ellis et al. 2012), improving the knowledge about the physiology of threatened species may help their conservation. The use of noninvasive techniques to quantify the physiological stress of wildlife has received great support, particularly when involving endangered species (Millsbaugh and Washburn 2004). This could be a promising tool for our target species given the high mortality rate of handled little bustards (Ponjoan et al. 2008). Determining the physiological response of little bustards using fecal glucocorticoid metabolites may give new insights on whether changing agricultural practices cause physiological stress and how organisms adapt to new environments with increasing human pressure. Because human pressure in agricultural areas may likely continue to increase, its effects on steppe birds of conservation concern should be carefully monitored to avoid potential negative impacts on their declining populations.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.behco.oxfordjournals.org/>

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