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Climate change and human population growth have increased anthropogenic threats to biodiversity and habitat fragmentation. Ecologists and conservationists need tools to assess the effect of these ecological and environmental perturbations on organismal fitness. One possibility is glucocorticoids (e.g. cortisol and corticosterone) which integrate various factors such as anthropogenic disturbances, predation, food or environmental stressors. Here we tested the hypothesis that fecal glucocorticoid metabolite concentrations (GCMs) in wild female elk *Cervus canadensis* increased as the hunting season progressed. We also examined the influence of year, food availability and elk group size on fecal GCMs. We found that as the hunting season progressed, fecal GCMs tended to decrease. We also found that as the number of cows in a group increased, GCMs decreased and found a strong effect of year on fecal GCMs, with samples collected in 2016 having lower fecal GCMs than those collected in 2015, 2017 and 2018. However, yearly variation was not driven by availability of hard mast forage. The association between hunting pressure and fecal GCMs and identifying what is driving yearly variation in fecal GCMs warrants further study. We highlight the negative influence of group size, possibly due to vigilance, on fecal GCMs and the importance of examining ecologically relevant covariates to accurately identify main treatment effects.

Keywords: anthropogenic stress, corticosterone, management, physiological ecology, predation

Hunting has long been engrained in many societies, with roles that range from cultural aspects to sustenance provisioning, and more recently, management of wildlife. Although evidence of the direct demographic impacts of hunting on wildlife is prevalent (Anderson and Burnham 1976, Topp-Jørgensen et al. 2009, Creel et al. 2011), theoretical advances in the ecology of fear (Brown et al. 1999, Laundré et al. 2010) also now highlight indirect impacts of hunting, such as changes in behavior and physiology (Bateson and Bradshaw 1997). These changes can affect various traits including antipredator behavior, immune function and reproduction (McEwen and Wingfield 2003, Romero et al. 2009), subsequently reducing population growth, in conjunction with direct removal of individuals (Andresen and Laurance 2007, Brook et al. 2012).

While there is considerable evidence of the physiological stress response on animals' physiology and behavior (McEwen and Wingfield 2003, Romero et al. 2009), how best to quantify individual stress levels is still debated. When animals experience an adverse event, a myriad of physiological and behavioral changes occur that are associated with the activation of the hypothalamic–pituitary–adrenal axis and subsequent release of glucocorticoids (e.g. cortisol and corticosterone; McEwen and Wingfield 2003, Romero et al. 2009). Glucocorticoids respond to a diverse range of stressors, integrating the stressors into a physiological response (Hardy et al. 2002, Malcolm et al. 2014, Graham et al. 2017). Additionally, environmental factors such as food availability and weather that vary across years (Boonstra et al. 2001, Gesquiere et al. 2008, Wingfield 2013), and ecological factors such as sociality and group size (Lima and Dill 1990, Caro 2005, Voellmy et al. 2014) can alter the magnitude of the

glucocorticoid response to a stressor. While short-term glucocorticoid increases can be an adaptive homeostatic response, long-term glucocorticoid increases can negatively impact an animal's health and reproduction (McEwen and Wingfield 2003, Romero et al. 2009). Consequently, glucocorticoids are being used in ecology and conservation as an index of the physiological state and health of an animal (Millspaugh and Washburn 2004, Busch and Hayward 2009). Non-invasive measures of glucocorticoids, such as fecal glucocorticoid metabolites (GCMs), typically integrate the stress response over 12–24 h in ungulates, enabling detection of signatures of stressors experienced over this timeline (Millspaugh et al. 2001, Möstl et al. 2005, Palme et al. 2005).

In wildlife studies, glucocorticoids can help us understand the direct effects of a stressor (e.g. predation, competition and resource limitation; McEwen and Wingfield 2003, Busch and Hayward 2009, Romero et al. 2009), and other confounding variables (Gesquiere et al. 2008, Wingfield 2013, Malcolm et al. 2014). For example, while human hunting directly elevates glucocorticoid concentrations (Bateson and Bradshaw 1997), glucocorticoids may also be influenced by group size, vigilance levels and forage availability (Lima and Dill 1990, Kitaysky et al. 1999, 2007, Caro 2005). For instance, glucocorticoids decrease with increasing forage availability for many species of birds (Kitaysky et al. 1999, 2007). While forage availability can vary between years, driving annual variation in glucocorticoids, so too can other environmental and ecological factors such as weather (Buck et al. 2007) and predator abundance (Boonstra et al. 1998). As such, measuring these potentially confounding variables increases the accuracy of measuring glucocorticoids as an indicator of anthropogenic impacts (e.g. hunting).

Here, we measured fecal GCMs levels in free-ranging female elk *Cervus canadensis* to examine effects of hunting, food availability, group size and annual variability of environmental factors to account for potential varying levels of fecal GCMs. Due to extirpation of key predators, elk populations can increase significantly, damaging plant communities and personal property, and increasing disease transmission between herds (Conover 2001, Walter et al. 2010, Sargeant et al. 2011). One population management tool is hunting, which promotes plant regeneration and ecosystem health (Conover 2001, Walter et al. 2010, Sargeant et al. 2011). However, it is unclear how hunting pressure affects elk physiology, and consequently reproduction, fitness and potential population dynamics. Therefore, understanding the indirect effects of hunting and ecological variables on elk physiology is important in identifying potential population-level impacts. Our research focused on a relatively small but naturally sustained elk population (~1100 animals) unevenly distributed across ~6500 km[2] in north-central Pennsylvania, USA. A brief but intense (6-day) elk hunting season provided an opportunity to examine the temporal influence of hunting activity on elk fecal GCMs. We predicted that hunting pressure (as measured by number of elk harvested and day of harvest) would increase fecal GCMs levels, and that fecal GCMs would have a negative relationship with food availability and group size.

Methods

Study area

The elk hunting season in Pennsylvania occurs in early November, running for six days (Monday–Saturday). Successful hunters must visit the check station with their elk within 24 h of harvest. The annual harvest is approximately 100 elk – about 10% of the overall population (1000–1100 elk; Banfield and Rosenberry 2020) and the number of hunter licenses are issued by hunt zone and correlated with elk population in each zone. Elk hunters began sample collection the first day of the hunting season and used a 50 ml screw-top tube and instructions given prior to the season to collect a sample of 3–5 fecal pellets from the rectum of their harvested elk while field dressing from 2015 to 2018. Pennsylvania Game Commission staff collected the samples at the elk check station. Staff kept fecal samples on ice (< 24 h) before freezing at −20°C for later analysis at Penn State University. Staff obtained dressed weight by a scale (± 1 lb) and estimated the live mass by adding 30% (dressed weight ×1.3; Blood and Lovaas 1966, Blood and Smith 1984).

Female elk are gregarious and generally remain in groups consisting of several cow–calf pairs. Hunters estimated and noted group size, or the number of elk observed with each harvested animal, in the field and reported at the elk check station. In our models we included 'harvested' and 'days of hunting' as two separate covariates to indicate hunting pressure. Harvested signifies the number of elk harvested per zone per year. Days of hunting represents the period (in days) between the start of the hunting season and the day a specific animal was harvested. Sample collection on the first day of the hunting season is represented with a value of zero and likely reflects baseline glucocorticoid samples as it takes 12–24 h for integration of blood glucocorticoids into elk feces (Millspaugh et al. 2001, Möstl et al. 2005, Palme et al. 2005). Hunting pressure should thus be reflected in samples collected on subsequent days, with glucocorticoid concentrations increasing from day 0 to day 6, if our hypothesis is supported. As there were no samples from day 6 and only three samples collected on day 5, we analyzed day 0 to day 4.

Pregnancy may increase glucocorticoid levels during gestation and the binding globulin for progesterone also has a strong affinity for glucocorticoids (Breuner and Orchinik 2002). Therefore, we determined pregnancy status for each female elk via an ELISA for serum pregnancy-specific protein B (PSPB; Noyes et al. 1997, Seixas et al. 2019) from blood samples collected from each female postmortem. Lactation was assessed by palpating the udder and visually confirming presence of milk at the check station. While we recorded reproductive characteristics (pregnancy and lactation) to account for individual sources of variation, we did not expect them to correlate with fecal GCMs as glucocorticoids are not elevated during early pregnancy but only during late pregnancy (Smith et al. 1973, Sandman et al. 2006). This is further supported by our sampling timeframe, which began approximately four to six weeks after the beginning of the rut for elk in North America when insemination occurs (Cook et al. 2004, Forrest and Clair 2009).

We used a hard mast index (HMI) to describe elk condition on an annual basis due to annual fluctuations in hard mast (Lupardus et al. 2011). Personnel from various agencies throughout Pennsylvania conducted an annual survey of hard mast species each year of our study area from 2015 to 2018 according to methods in Noyce and Garshelis (1997). Briefly, survey participants who were regularly in the field and familiar with local tree species identified subjective assessments for hard mast produced by red *Quercus rubra* and white oak *Q. alba*. Previous research identified 9–50% of elk and red deer *Cervus elaphus* diets were comprised of acorns which provides a high-quality source of protein and other dietary requirements throughout the range of this genus (Jedrzejewski et al. 2006, Heffernan 2009, Lupardus et al. 2011). Participants made two categorical subjective assessments of each species based on abundance and productivity. Abundance was rated as 0 (absent or scarce), 1 (uncommon), 2 (common), 3 (abundant) or 4 (very abundant). We rated productivity as 0 (little or no fruit produced), 1 (below average), 2 (average), 3 (above average) or 4 (bumper crop). We determined these values by averaging over each survey participant (2–5) per county for each year. We considered both to be an appropriate index for forage condition so we multiplied abundance by productivity for each species of oak, then averaged the results from each county together, and used the totals as the final estimate of HMI for each year (average annual HMI).

After determining average annual HMI, we created a kriged surface across the state for each year in program R (<www.r-project.org>). We kriged averages for each county available from our participants using inverse distance weighted interpolation in the *gstat* package. We then geo-referenced the location of each sampled elk and extracted HMI from the kriged surface raster at 30 m resolution for the corresponding year of harvest prior to modeling (Fig. 1).

Fecal sample preparation

We thawed fecal samples and placed them into a lyophilizer for two days to remove moisture for hormone extraction. We weighed samples until they no longer lost mass to ensure complete desiccation. We then ground each sample, while removing any large particulates (seeds, undigested flora), and mixed thoroughly. This method preserves fecal GCMs and allows for homogenization of the sample prior to extraction (Wasser et al. 1996). We extracted the GCMs from the homogenized feces following Millspaugh et al. (2001). We placed 200.2 ± 2.4 mg of dried feces in 13×100 mm borosilicate tubes with 2.0 ml 90% methanol and vortexed at high speed (~2000 rpm) on a multi-tube vortexing rack at room temperature for 30 min. We centrifuged samples at 8000 g at room temperature for 20 min. We then pipetted off the supernatant and stored it in 2.0 ml microcentrifuge tubes at −20°C for analysis.

Fecal glucocorticoid metabolite assays

To quantify fecal GCMs, we used MP Biomedical ^{125}I Corticosterone radioimmunoassay kit (no. 07-120103, Santa Ana, CA). This assay has been previously validated for use in elk using adrenocorticotropin challenge and parallelism (Millspaugh 1999). We assayed samples in duplicate following manufacturer's guidelines except that we halved the volume of all reagents following Millspaugh et al. (2001). We ran samples over four assays and the samples fell within the detectable range of the standard curve. Inter-assay variation was 7.53% and the average intra-assay variation was 5.87%.

Data analysis

All analyses were conducted in program R using linear mixed models (<www.-r-project.org>, package: *lme4*).
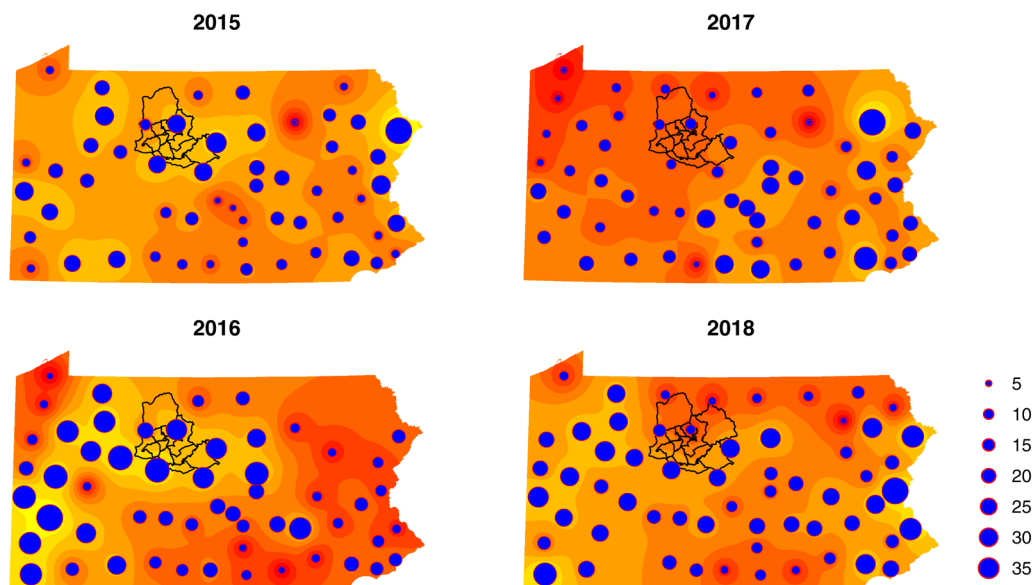

Figure 1. Hard mast index across Pennsylvania from 2015 to 2018 using annual estimates from personnel within each county. The graduated symbols reference the range of the hard mast index from a low (5) to high score (35) for a given county. The polygons reflect locations of the elk harvest zones within elk range during the respective year of a hunting season.

To ensure assumptions of the model were met, fecal GCMs concentrations and group size was natural log transformed for normality. We examined a correlation matrix for all covariates before modeling to screen for collinearity. We could not include herd size and harvested in the same model as they were strongly collinear. We included fecal GCMs as the dependent variable and herd size or harvested, days of hunting, lactation status, pregnancy status and year as independent variables in the full model. We included harvest zone as a random effect for all models. We ran combinations of different variables to address our main question of the impact of ecological and hunting variables on fecal GCMs and then also tested for effects of each of these variables independently (Table 1). We used second order bias correction for Akaike's information criterion (AICc; Burnham and Anderson 2002) to select the most parsimonious model among the suite of models. We present results as model coefficient estimates (Estimates) ± 1 SE with 95% confidence intervals (CI). Lastly, as PSPB has lower sensitivity during early gestation (Seixas et al. 2019) and neither pregnancy nor lactation altered the model selection (ΔAIC >100), we removed them from further analysis.

Results

Staff collected elk fecal samples from 181 free-ranging female elk in north-central Pennsylvania in 2015 (n = 34), 2016 (n = 51), 2017 (n = 53) and 2018 (n = 43). Harvest numbers declined considerably from day 0 to day 4 with 111 (61%), 31 (17%), 14 (8%), 18 (10%) and seven (4%) elk harvested, respectively.

The model with the most support indicated that days of hunting, group size and year influenced fecal GCMs of adult female elk (AICc Weight: 0.81, Table 1). Models with HMI removed received more support than models with HMI (Table 1). Models with group size had more support than models with harvested (Table 1).

While we observed a negative relationship between fecal GCMs and days of hunting, the 95% CI overlapped zero and was therefore not supported (Table 2). Elk from larger groups had lower fecal GCMs (Table 2, Fig. 2). Year had a strong influence on fecal GCMs, with elk from 2016 having the lowest concentration (Table 2, Fig. 2) while 2015, 2017 and 2018 had higher concentrations (Table 2, Fig. 3).

Discussion

Contrary to our expectations, fecal GCMs did not increase with hunting pressure (number of elk harvested or number of days into the hunting season) in our study system. Fecal GCMs tended to decrease with hunting pressure (days of hunting), and as herd size increased, fecal GCMs decreased. We also found large differences in fecal GCMs between years, however, HMI was not driving these trends. While previous studies have found no effect of predation (i.e. wolves; Creel et al. 2009) on fecal GCMs in ungulates, our results extend this finding to an ungulate species that experienced anthropogenic hunting pressure. Hunting pressure does not appear to elevate stress as indicated through fecal GCMs. Furthermore, annual variation, likely from environmental factors other than food availability, such as precipitation or temperature, plays a role in fecal GCM concentrations.

We found no evidence of increased fecal GCMs with the number days of hunting. We had predicted that fecal GCMs would increase with this index of hunting pressure, as previously shown in red deer (Bateson and Bradshaw 1997). However, sampling in this earlier study involved long chases of elk (average 19 km) using hunting dogs, and the elevated glucocorticoids in blood samples likely reflected this recent chasing event (Norum et al. 2015). In our study, hunters may have harvested an elk soon after observing it on the first day without use of dogs or actively driving the elk (Bateson and Bradshaw 1997, Norum et al. 2015). This allowed us to examine the indirect and prolonged impacts of hunting pressure on elk, as indicated by fecal GCMs, as opposed to direct, short-term hunting impacts shown in sera (Millspaugh and Washburn 2004). Additionally, we measured glucocorticoid metabolites in fecal samples which should have integrated glucocorticoids over the previous 12–24 h, potentially masking any acute stress caused immediately prior (<12 h) to a harvest (Millspaugh et al. 2001, Möstl et al. 2005, Palme et al. 2005). While subsequent days to a hunting attempt would likely have had adequate time to elevate fecal GCMs if a stressor was occurring, the lack of an effect of hunting day would indicate that either the elk collected on days 2–4 did not experience a harvest attempt, that any harvest attempts did not lead to an increase in fecal GCMs, or all elk experienced a stressor prior to the hunting season which would mask the impact of the hunting season (Millspaugh et al. 2001, Möstl et al. 2005, Palme et al. 2005).

Table 1. Top models using Akaike's information criteria (AICc) adjusted for small samples size for examining the impacts of variables on fecal glucocorticoids during the hunting season 2015–2018 in adult female elk. Fixed effects included year, days of hunting (days), hard mast index (HMI), number of cows in the group at the time of collection (cows), and number of elk harvested per year per zone (harvested). The random effect of harvest zone (1|Harvest zone) was included in all models.

Model terms	AICc	ΔAICc	AICc weight
Year + Days + Group size + ------ + 1|Harvest zone	1743.36	0.00	0.81
Year + Days + ------------ + HMI + 1|Harvest zone	1747.38	4.02	0.11
Year + Days + Harvested + HMI + 1|Harvest zone	1748.97	5.61	0.05
Year + ------ + ----------- + HMI + 1|Harvest zone	1750.47	7.10	0.02
Year + ------ + ----------- + ------ + 1|Harvest zone	1752.80	9.44	0.01
Year + ------ + Harvested + ------ + 1|Harvest zone	1754.78	11.42	0.00
------ + ------ + ----------- + HMI + 1|Harvest zone	1822.36	79.00	0.00
------ + Days + ------------ + ------ + 1|Harvest zone	1901.48	158.12	0.00
------ + ------ + Group size + ------ + 1|Harvest zone	1901.92	158.56	0.00

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Table 2. Parameters, model coefficients (Estimates), standard error (SE) and 95% confidence intervals (CI) for the top model for examining the impact of different factors on adult female elk fecal glucocorticoids.

Parameter	Estimates	SE	CI
Intercept	119.641	7.792	104.611 to 134.746
Year (2016)	−52.520	7.085	−66.374 to −38.919
Year (2017)	33.623	6.918	20.176 to 46.994
Year (2018)	22.994	7.157	9.036 to 36.766
Days	−3.271	2.011	−7.118 to 0.673
Group size	−5.266	2.157	−9.521 to −1.181

We found a negative relationship between fecal GCMs and group size. As group size increases, individual vigilance often decreases (Lima and Dill 1990, Caro 2005, Voellmy et al. 2014), as do glucocorticoids (Lima and Dill 1990, Caro 2005). Alternatively, as group size and the number of elk harvested per zone were collinear, the negative relationship between group size and fecal GCMs could be due to the temperament of harvested elk, reflected in changes in glucocorticoids (Found 2015). For example, personality, as measured along the bold–shy continuum, can influence glucocorticoid levels, with shyer animals having higher glucocorticoids and vigilance than bolder individuals (Clary et al. 2014). If shyer animals were more wary of predators, they may have left the area at the start of the hunting season to escape hunting pressure (Root et al. 1988), as suggested by
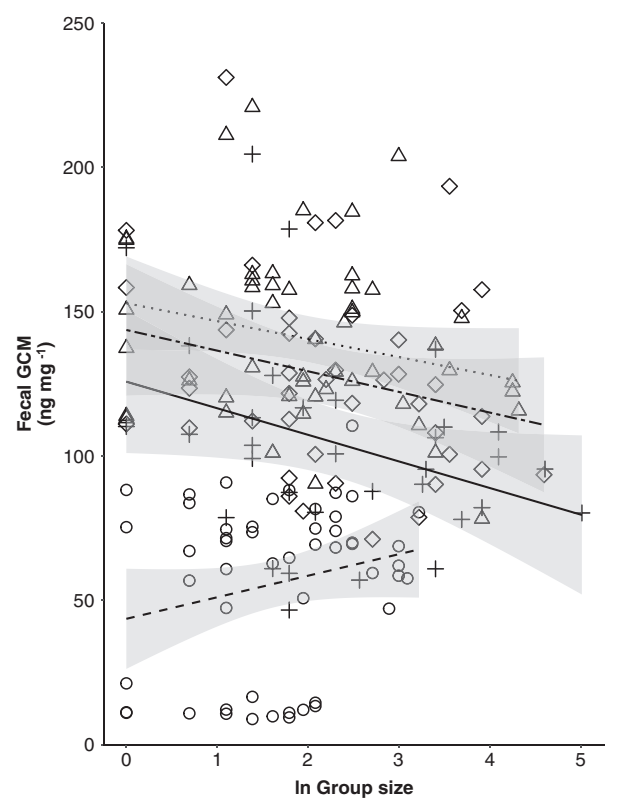

Figure 2. The relationship between fecal glucocorticoid metabolites (fecal GCMs) and natural log of cows per group (group size) in 2015 (+ and dash-dot line), 2016 (○ and dashed line), 2017 (Δ and solid line) and 2018 (◇ and dotted line) in female elk (n = 181). Each symbol represents a single elk. The best-fit regression line is shown and the standard error is shaded grey.
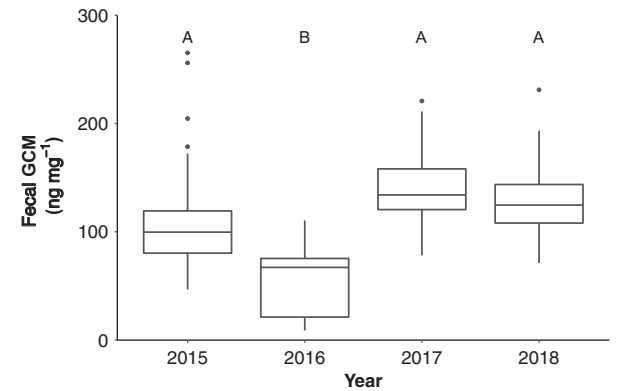

Figure 3. The relationship between fecal glucocorticoid metabolites (fecal GCMs) and year (2015 n = 34; 2016 n = 51; 2017 n = 53; 2018 n = 43) in female elk. Box plot represents median, 25–75 quartile range and range with dots representing outliers. Box plots bearing different letters had non-overlapping 95% confidence intervals.

changes in habitat use and foraging in elk due to natural and anthropogenic pressures (Conner et al. 2001, Creel et al. 2009, Cleveland et al. 2012). As bolder animals have lower glucocorticoid levels and may have a higher threshold to what is perceived as stressful (Rupia et al. 2016), this would leave animals with lower glucocorticoid levels to be harvested, leading to a perceived decrease in population glucocorticoid levels as opposed to the actual change in population demographics. While cow group size had a negative relationship on fecal GCMs which was most likely due to vigilance, we cannot exclude the correlation between cow group size and hunter density. If this result was driven by hunter density, however, we would have expected a positive relationship between cow group size and fecal GCMs. As such, further research is needed to separate the impacts of herd size and hunter density. Additionally, while our findings based on model selection supported that fecal GCMs decreased with group size, but not with the number of elk harvested per zone, the influence of elk personality in response to hunting pressure warrants further investigation.

While we focused on anthropogenic disturbance (days of hunting), the unrelatedness between predation/hunting pressure and glucocorticoids in elk has been documented in other ruminants. For instance, increased predation risk due to higher wolf density did not increase fecal GCMs in elk (Creel et al. 2009), and the largest source of anthropogenic disturbance found in one elk population was from recreational activities other than hunting (e.g. tourism and snowmobiling; Millspaugh et al. 2001). In this study, fecal GCMs may lack association with hunting pressure due to the short and infrequent nature of the hunting seasons for elk (once a year, < 6 days long; Millspaugh et al. 2001). Alternatively, these brief hunting episodes may impact behavior more than physiology, evidenced by decreases in foraging patterns and movement away from hunters (Millspaugh et al. 2000, Winnie and Creel 2007, Creel et al. 2009). While predation risk from natural predators can decrease foraging and reproduction (Creel et al. 2009), hunting is unlikely to cause a pronounced impact on elk reproduction through the hypothalamic–pituitary–adrenal axis due to the short nature of the hunting season and the harvest event. The impact

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of hunting on elk behavior, however, and its connection to altered foraging and reproduction, warrant further examination.

We found a strong influence of year on fecal GCMs in female elk. Time of the year and seasonality are strong drivers of glucocorticoids in many animals (Waas et al. 1999, Place and Kenagy 2000, Boonstra et al. 2001), however, our samples were collected at the same life history stage each year. Therefore, lower concentration of fecal GCMs in 2016 was likely driven by environmental or biotic factors such as weather or forage availability (Gesquiere et al. 2008, Wingfield 2013). However, our measure of forage availability, HMI, did not describe variation in fecal GCMs. During autumn, woody plants are the dominant forage class for elk in some areas (Pennsylvania: Heffernan 2009, Tennessee: Lupardus et al. 2011). While oaks are the dominant woody species consumed during autumn, this typically makes up 9–50% of elk diet (Jedrzejewski et al. 2006, Heffernan 2009, Lupardus et al. 2011). Understanding the importance of legume, graminoid and forb availability in the elk diet may help us link forage availability and fecal GCMs. Other environmental factors such as long-term weather data or recreational use of elk habitat could be considered in future studies to identify the driver of annual variation.

Conclusion

Fecal GCMs concentrations in this study were not elevated by hunting pressure, potentially allowing elk to avoid important fitness-relevant costs of elevated glucocorticoids (e.g. decreased reproduction or immune function; McEwen and Wingfield 2003, Romero et al. 2009). While the number of elk harvested per zone was negatively correlated with fecal GCMs, this is most likely due to group size and vigilance, which is common for this gregarious species. Future research parsing out the influence of group size and/or vigilance behavior would be a valuable addition to the literature on fecal GCMs in this species. Annual variation in fecal GCMs would suggest that some unmeasured environmental or biotic variable was influencing fecal GCMs levels in our elk population. For example, an unusually low snowfall occurred in winter 2015–2016 which coincided with the year we documented the lowest fecal GCMs in elk for any of the four years we collected data. Further research into environmental factors, such as weather and other forage availability, could provide important details into potential drivers of the annual variation in fecal GCMs.

Data availability statement

Raw data for fecal glucocorticoid metabolites measured in elk is open access and available at Zenodo.org at <https://doi.org/10.5281/zenodo.3856427>.

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