WILDLIFE BIOLOGY

Short communication

Hunting method affects cortisol levels in harvested mountain hares (*Lepus timidus*)

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Wildlife Biology
2024: e01366
doi: 10.1002/wlb3.01366
Subject Editor: Luca Corlatti
Editor-in-Chief: Ilse Storch
Accepted 05 October 2024

The direct effects of hunting on hunted individuals and populations have been well known for a long time. However, recently there has also been an increased focus on the indirect, non-lethal effects of hunting. When approached by a possible threat such as a predator, the prey releases various stress hormones into the bloodstream. Cortisol is one of these hormones and the blood concentration is an indicator of stress levels in mammals. Here we report on a study on the effects of using hunting dogs versus walk-up shooting on mountain hare blood cortisol levels. We sampled 20 hares hunted using dogs and 32 control hares hunted without using dogs. On average the cortisol level in hares hunted using dogs was 44.6 ng/ml, while in hares harvested without being chased by dogs it was 6.8 ng/ml. Based on the blood hormone levels of this study we cannot conclude if the elevated cortisol levels we see in the hares hunted using dogs was harmful to the hares had they not been shot. However, given what is known about the effects of chronic stress, we would caution against repeated chases of individual hares. The cumulative effect of stressors including hunting is likely crucial for any effects on reproduction and survival. Thus, there is a need to evaluate the long-term effects of hunting chases and other human activities on mountain hare stress hormone levels, and to investigate the long-term effect on hare behavior, space use, survival, reproduction and recruitment.

Keywords: Animal welfare, blood sampling, harvesting, hounds, hunting dogs

Introduction

Hunting hares (*Lepus* spp.) with dogs has a long tradition in Europe and was reported from ancient Greece as long ago as 2000 years before the present (Blane 1788). In

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Norway, hunting with dogs, as well as the breeds of dogs used in such hunting (hounds), were introduced from Germany in the 16th century. For several hundred years, hunting mountain hares (*Lepus timidus*) with hounds was mostly carried out by the aristocracy with traditional hunting parties hosted at large estates. For example, the Norwegian national hero and polar explorer Fridtjof Nansen, Norway's King Haakon V and other celebrities attended hunting parties as late as 1925 (Barikmo and Pedersen 1997). During the 18th and 19th centuries, hunting hares with hounds became common among the middle class and landowning farmers (Barth 1891). Thus, hunting hares with hounds has been part of Norwegian culture for more than 400 years.

In Norway, hunting hares using hounds normally starts in the morning, with hounds being released in the forest, where they start to track the hare's path from the previous night's activity. If the hound finds a hare it will start barking (Norwegian: *los*, from German: *los*) and follow the hare. The hunter(s) hear the location of the hound, and position themselves in areas where they think it is likely that the hare will run past and wait for an opportunity to shoot. During the los the hound is normally 2–15 minutes behind the hare (Barikmo and Pedersen 1997). The hound may lose the track of the hare (Norwegian: *tap*) as the hare runs in the same tracks multiple times, crossing roads, changing direction, etc. These breaks may last for any length of time from a few minutes to several hours, until the dog finds the hare again, the hunter decides to end the hunt, or the hound gives up. In addition to actual hunting, harehound owners also train their hounds in finding and pursuing hares. Thus, a hare may be chased multiple times without being shot.

Along northern coastal areas of Norway, hares may reach high densities on islands free of mammalian predators. In such locations, hares may be hunted without using hounds, either by flushing and shooting them with a shotgun, or spotting them at a distance and shooting with a rifle. The majority of Norway's annual game bag of hares is harvested as by-catch from bird hunts, which represent a much larger number of hunters compared to hare-specialist hunters using hounds (Pedersen and Pedersen 2012).

The welfare of hunted game animals is increasingly raised by animal rights organisations as a social–political issue exercising moral and ethical discourse (Allen et al. 2023). The subject is also of interest to wildlife biologists, exemplified by the current special issue in Wildlife Biology. There will always be subjective debate as to whether any level of discomfort, stress or pain is acceptable when harvesting animals. Quantifying potential discomfort provides a means to understand the impacts of hunting. The direct effect of mortality on hunted individuals and populations have been well known for a long time (Barth 1891, Leopold 1933). However, more recent research includes a focus on indirect, non-lethal effects, mostly for large mammals (Skjørestad 2018, Støen et al. 2018, Græsli et al. 2020), but research on small game is limited (see, however, Olsson et al. 1996, Brøseth and Pedersen 2010).

When approached by a possible threat such as a predator, the sympatric nervous system of the prey activates and releases quick-acting hormones into the blood, initiating a 'fight-or-flight' response (Reeder and Kramer 2005). Simultaneously, the slower endocrine response is activated, releasing a series of slower-acting hormones such as cortisol. These hormones take minutes to hours to impact an individual's physiological state – such as cognitive ability, immune system function and reproduction – and the length of these altered physiological states vary (Boonstra 2013, Kelm et al. 2016). If the impact of such disturbance is sufficient, changes in condition, fecundity, offspring quality, movements and survival may be expected.

Levels of stress, and especially long-term stress, can have a significant impact on the wellbeing of an animal. While predictable environmental changes, i.e. seasonality, would be unlikely to cause significant stress to animals, increased exposure to unpredictable changes can contribute significantly to chronic stress (McEwen and Wingfield 2003, Reeder and Kramer 2005, Boonstra 2013). Stress hormones are vital in the regular functioning of an animal. Yet, the daily levels of stress hormones are tightly controlled in most animals, as prolonged increases in stress hormones contribute to a variety of negative impacts, such as an increase in cellular damage and a decrease in immune function (McEwen and Wingfield 2003). However, previous studies on chronic stress have often focussed on laboratory animals, whereas it is possible that chronic stress in some wild animals is adaptive and enables them to persist despite the stressors (Boonstra 2013). Regardless, many animals are now experiencing more frequent and prolonged exposure to stressors and even those adapted to chronic stress may experience maladaptive responses. Long-term stress can affect subsequent generations, for example through effects on the immune function, affecting survival and reproduction, and thus, a population.

Physical activity may increase levels of stress hormones, e.g. with the mating season coinciding with a peak in glucocorticoid metabolites in mountain hares (Rehnus and Palme 2021), and in other animals (Edwards and Boonstra 2018). With the already high levels of stress during reproduction any additional stressors are likely to have negative implications to survival and reproductive success. For example, in pregnant snowshoe hares (*L. americanus*) experiencing significantly increased glucocorticoid concentrations from exposure to a predator, there was not only a reduction in the number of offspring they produced, but also in the quality of their offspring (Sheriff et al. 2009). For male snowshoe hares, testosterone levels are lower in years of high predation as they are being supressed by higher levels of glucocorticoids (Boonstra 2005).

Hunting using hounds may increase stress to harvested animals as compared to other methods of hunting such as walk-up shooting. To ensure that hunting is carried out in a way that does not cause unnecessary discomfort, there is a need to evaluate the non-lethal disturbance effects to the hunted animals. This study is, as far as we know, the first attempt to evaluate stress levels in hares harvested using hunting with hounds. The specific objective was to quantify blood cortisol levels in hares harvested using 1) hunting with hounds involving a pursuit and 2) walked-up shooting of hares from their daily forms. We hypothesised that blood cortisol levels would be higher in hares harvested by hounds, and that levels would rise with increased duration of the chase.

Methods

Field sampling

Hunted hares were sampled across Norway opportunistically during the normal annual hunting season from 10 September through to either the end of February or 15 March (depending on area).

Hares were sampled in 2020–2022 with a total of 20 hares shot after being hunted by hounds (hound samples) and 32 shot after no pursuit by hounds (control samples). These control hares were shot either following a short sprint after flushing or shot while still sitting in their form. Twenty-six of these control hares were known to be resting in their day-forms prior to shooting. For samples of known sex, there were 24 females and 21 males.

Blood samples were taken either directly from the heart (if that was blood filled), or from the chest cavity immediately after shooting in 15 ml vacutainer EDTA vials. Sampling from the chest cavity rather than the abdominal cavity minimized possible contamination from gut content; however, any samples visually contaminated by gut content were excluded from further analysis. Blood samples were kept unfrozen in the field, and brought back to the lab at the end of the day, where the serum was collected, frozen and stored at −20°C for further hormone analysis.

During hare hunting (both with or without hounds), hares may be aware of the hunter in the minutes or seconds before being shot. This may affect the immediate action of the sympatric nervous system; however, as we are analysing hormone levels from the slower-acting endocrine system, levels of cortisol will not be affected by any response to the hunter immediately before shooting. Thus, cortisol will reflect stress levels on a timescale of several minutes before the hare was shot, i.e. stress levels at the time of the hounds' chase, or prior to any flushing from the hare's form. Thus, our control group should represent hare baseline resting cortisol levels.

Lab analysis

To determine total serum concentration of cortisol, we used a chromatographic technique (SFC-MS/MS) instead of the more traditional immunological assay. While immunological assays are known for their good sensitivity, they frequently suffer from a lack of specificity due to cross-reactivity with other steroids and their metabolites (Stanczyk et al. 2007, Sait et al. 2023). Chromatographic techniques consistently report lower concentrations than immunological assays when analysing steroid hormones (Faupel-Badger et al. 2010). This discrepancy can be attributed to the cross reactivity in immunological assays, which often leads to overestimated blood steroid concentrations – a phenomenon extensively documented for cortisol (Lewis and Elder 1985, Wood et al. 2008, Dodd et al. 2014, Krasowski et al. 2014). To avoid interference from other steroid hormones or metabolites, causing possible overestimation, we determined total serum concentration of cortisol using supercritical fluid chromatography tandem mass spectrometry (SFC-MS/MS) according to Sait et al. (2023) with minor modifications.

In short, we transferred serum (150 µl) to 15 ml polypropylene tubes, followed by the addition of 450 µl of methanol containing 0.1% (w/v) ammonium formate. After vortex-mixing and centrifugation at room temperature (4000 rpm for 10 min), the supernatants were passed through pre-conditioned HybridSPE cartridges (30 mg/1 ml) before analysis with SFC-MS/MS. The SFC-MS/MS analysis was performed using a Waters ACQUITY ultraperformance convergence chromatographic (UPC^2) system coupled to a triple quadrupole mass spectrometer with a Z spray electrospray ionization (ESI) source. Separation was performed with a Viridis® CSH Fluoro-Phenyl UPC^2 column (130 Å, 1.7 µm, 2.1 mm × 100 mm) with a Viridis® CSH Fluoro-phenyl VanGuard™ pre-column (2.1 mm × 5 mm). Supercritical CO_2 (A) and MeOH with 30 mM ammonium acetate (B) were used for chromatographic separation. The gradient elution began with 2% B for 0.5 min, increased to 17% B over 2.5 min (held for 0.5 min), then returned to 2% B over 0.5 min (held for 1 min), for a total run time of 5 min. A flow rate of 1 ml/min, with an injection volume of 1 µl was employed. To improve ionization, we utilized a post-column make-up flow of MeOH/IPA with 0.1% v/v formic acid at a flow rate of 0.2 ml/min. For further details, see Sait et al. (2023).

Quantification of serum cortisol was accomplished by use of internal standard (corticosterone-13C3) and with matrix-matched calibration standards prepared by spiking cortisol into the matrix prior to extraction (Asimakopoulos et al. 2014). The extraction efficiency was evaluated by calculating the absolute recovery (36.4%) and relative recovery (92.6%) for cortisol at a concentration of 20 ng/ml. Method precision, assessed in triplicate at 20 ng/ml, demonstrated acceptable reproducibility, with a relative standard deviation (RSD) of 6.0%. The method's limit of detection (LOD) was determined at 0.417 ng/ml. Although the method performance was satisfactory, this yielded a relatively high detection limit.

Statistical analysis

In total, nine of the samples had cortisol levels below LOD, possibly due to the high specificity towards cortisol (see above). For these samples, we replaced this with an estimated value of 50% of the LOD value for analysis (0.208 ng/ml). Following the recommendations by Halsey (2019), we applied the data analysis with bootstrap-coupled estimation (DABEST) approach (Ho et al. 2019) for two group differences.

Results

Mean serum cortisol levels in hares hunted using hounds (44.6 ng/ml) was 6.6 times higher than that of the control group shot without being chased by hounds (6.8 ng/ml), the unpaired mean difference between the groups was 37.8 (95% CI: 24.9–59.5, Fig. 1a). For both groups, we had one outlier observation with extremely high levels of cortisol: both outliers were approximately five times higher than the cohort mean

(Fig. 1a). For the outlier individual in the control group we observed a large infection approximately 3 × 3 × 5 cm within the abdominal cavity that could explain the elevated cortisol levels. The cause of the increased values for the individual in the hound harvested group could not be explained as no discrepancies were noted. We found a mean cortisol value of 21.4 ng/ml for all hares. Males had a serum cortisol level of 23.2 ng/ml which was 1.7 times higher than females at 17.9 ng/ml, and the unpaired mean difference between males and females was 6.3 (95% CI: 7.5–23.6, Fig. 1b). For the hares shot after being pursued by a hound, mean duration of los before the hare was shot was 51 min (range 10–120 min). Cortisol concentrations increased initially (Fig. 2), but due to low sample size and large variation in cortisol concentration at the longer los durations, we cannot conclude how cortisol values are affected by chase duration.

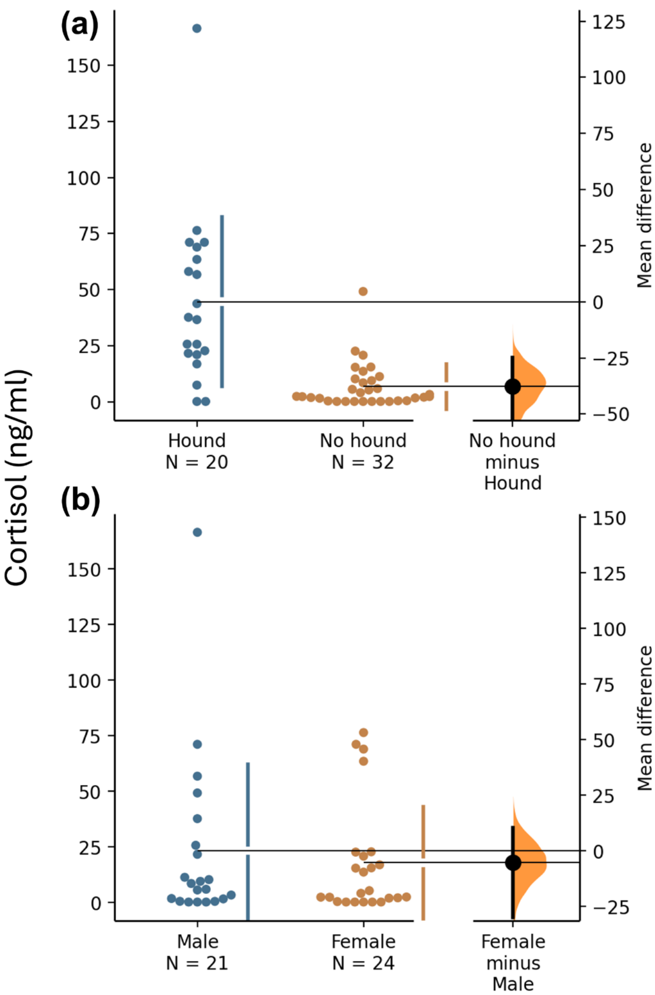

Figure 1. Mean difference in blood cortisol levels of (a) hares harvested using hounds versus without using hounds, and (b) males and females. Cortisol values in groups are plotted on the left axis; the mean difference is plotted on a floating axis on the right as a bootstrap sampling distribution (orange). The mean difference is depicted as a black dot, with the 95% confidence interval indicated by the ends of the vertical black error bar.

Discussion

This is the first study of blood cortisol levels in mountain hares, and consequently there are no within-species reference studies to compare with. However, Gentsch et al. (2018) found relatively small among-species differences in blood cortisol levels for European deer species. Therefore, comparing blood hormone levels of mountain hares with those of related hare species could provide valuable insights. In a study of live-caught snowshoe hares, Lavergne et al. (2021) found considerable inter-annual variation in cortisol levels in blood plasma, with mean population levels ranging from approximately 65 to 105 ng/ml across seven years. This is considerably higher than our mean value of 21.4 ng/ml. In contrast, Paci et al. (2006) investigated cortisol levels in European hares (*L. europaeus*) captured for translocation, and found levels ranging from 9.3 to 15.3 ng/ml. Despite the limited number of studies, the significant variation between species highlights the need for caution when comparing plasma hormone concentrations across different species. Furthermore, the environmental conditions (e.g. food availability, predator density, season of the year) under which the different species samples were collected would also have affected the hormone concentrations.

In a similar study design to the current study, Bateson and Bradshaw (1997) found average levels of 71 ng/ml plasma cortisol levels for red deer (*Cervus elaphus*) harvested using hounds, while undisturbed red deer harvested with a clean shot to the head or neck had mean cortisol levels of < 1 ng/ml. When investigating the effects of hunting method on roe deer (*Capreolus capreolus*) in Europe, Gentsch et al. (2018) found blood cortisol levels 4.9 ng/ml for roe deer harvested during stalking (comparable to our control group), 20.9 ng/ml for roe deer harvested during driven hunts and 14.2 ng/ml for roe deer harvested using dogs (comparable to our hound-hunted hares). Additionally, Gentsch et al. (2018) found that cortisol levels increased with chase duration.

Many studies use non-invasive fecal cortisol metabolite measurements as a measure of stress levels (Sheriff et al. 2011). Sheriff et al. (2010) found there to be good correlation between blood plasma cortisol levels and fecal cortisol metabolite levels.

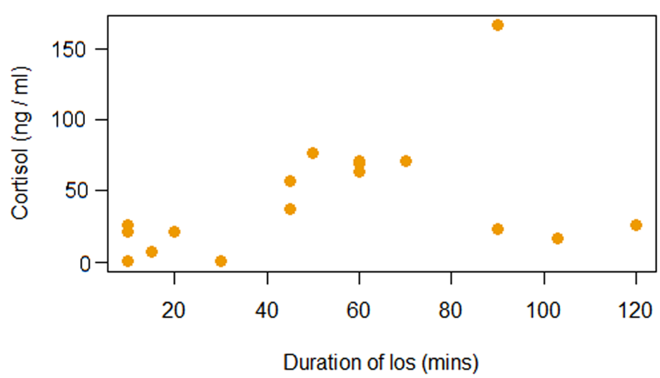

Figure 2. Duration of the los (hound pursuing the hare while barking) (in minutes) versus blood cortisol concentration in hares harvested using hounds.

Using fecal cortisol metabolites, Boudreau et al. (2019) investigated the effects of exposing snowshoe hares to experimental predation using dogs. Hares were chased three times per week over several months. They found an increase in fecal cortisol metabolites after exposing the hares to the simulated predator. They did not, however, find that hares exposed to experimental predators had any lower condition compared to control individuals (Boudreau et al. 2019). In contrast, a study on captive snowshoe hares found that females exposed to simulated predators had higher fecal cortisol metabolite concentrations. Consequently, these females had lower offspring size and body mass compared to control females (Sheriff et al. 2009).

The relatively high detection limit found for cortisol poses challenges for quantifying low concentrations of steroid hormones in biological samples. We suggest implementing an additional concentration step during extraction may help improve detection limits in future analyses. Further, we only measured total cortisol in plasma and did not measure cortisol levels downstream of the plasma, i.e. free cortisol. It is known that this free cortisol is what can inform us of how increased stress impacts different physiological traits in an animal, such as immune functioning and oxidative stress (Breuner et al. 2013).

Based on the blood hormone levels of this study we cannot conclude if the elevated cortisol levels we see in the hares hunted using hounds was detrimental to the hares. However, given what is known about effects of chronic stress and/or repeated exposure to acute stress, we would caution against repeated chases of individual hares. In addition to hunting, other forms of human disturbance may cause increased stress to hares. Rehnus et al. (2014) found increased levels of fecal glucocorticoid metabolites in mountain hares exposed to high skiing activity. The cumulative effect of stressors is likely crucial for any effects on reproduction and survival. Thus, there is a need to evaluate the long-term effects of hunting chases and other human activities on mountain hare stress hormone levels, and to investigate the long-term effect on hare behavior, space use, survival, reproduction and recruitment. A recent study of hares chased by dogs during coursing in Ireland, where the animal is not killed, suggested impacts on space use were limited to a few days immediate after coursing (Reid 2023). The animals settled into movement patterns similar to control animals shortly after the coursing event, with limited apparent impact on long-term mortality (Reid 2023). While field sports undoubtedly harvest animals, and it may be that such animals experience higher stress hormones during the hunt, it must also be acknowledged that – along with culture and tradition – field sports organizations and those individuals participating may positively contribute to conservation of hunted populations to support the sustainability of their sport, simultaneously benefiting the wider environment (e.g. through conservation of suitable habitat) (Reid et al. 2010). Thus, the issue of the impact of field sports on wild individuals and their populations is complex and warrants further study.

Acknowledgements – We thank the Norwegian Hunters and Anglers Association (NJFF) and their members for their cooperation. Roy Ågedal, Maik Rehnus and Webjørn Svendsen participated in project planning and sample collection. We would also like to thank the numerous hunters and their hounds who participated in sample collection, as well as landowners for providing access to study areas.
Funding – SP, TMC and STLS were funded by the Norwegian Environmental Agency (project no. 2020/1121) and the Trygve Gotaas Fund.
Permits – All hunters held legal permits to hunt and 1) passed a hunter's education course, 2) paid an annual hunting fee to the state of Norway, and 3) held landowners' permission to hunt on their land. This study required no further ethical permit under Norwegian law since sampling of the animals was done during the normal hunting season with all the necessary permits.

Author contributions

Simen Pedersen: Conceptualization (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Resources (equal); Visualization (lead); Writing - original draft (lead); Writing - review and editing (equal). **Tomasz M. Ciesielski:** Funding acquisition (supporting); Investigation (equal); Methodology (equal); Writing - review and editing (equal). **Clare Stawski**: Methodology (equal); Writing - original draft (supporting); Writing - review and editing (equal). **Neil Reid:** Writing - original draft (supporting); Writing - review and editing (equal). **Shannen T. L. Sait:** Investigation (equal); Methodology (equal); Writing - review and editing (equal). **Hans C. Pedersen:** Conceptualization (supporting); Funding acquisition (supporting); Methodology (supporting); Resources (equal); Writing - original draft (supporting); Writing - review and editing (equal).

Data availability statement

Data are available from the DataverseNO Digital Repository: https://doi.org/10.18710/7PFTWY (Pedersen et al. 2024).

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