

Heavily hunted wolves have higher stress and reproductive steroids than wolves with lower hunting pressure

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Summary

1. Human-caused harassment and mortality (e.g. hunting) affects many aspects of wildlife population dynamics and social structure. Little is known, however, about the social and physiological effects of hunting, which might provide valuable insights into the mechanisms by which wildlife respond to human-caused mortality.

2. To investigate physiological consequences of hunting, we measured stress and reproductive hormones in hair, which reflect endocrine activity during hair growth. Applying this novel approach, we compared steroid hormone levels in hair of wolves (*Canis lupus*) living in Canada's tundra–taiga ($n = 103$) that experience heavy rates of hunting with those in the northern boreal forest ($n = 45$) where hunting pressure is substantially lower.

3. The hair samples revealed that progesterone was higher in tundra–taiga wolves, possibly reflecting increased reproductive effort and social disruption in response to human-related mortality. Tundra–taiga wolves also had higher testosterone and cortisol levels, which may reflect social instability.

4. To control for habitat differences, we also measured cortisol in an out-group of boreal forest wolves ($n = 30$) that were killed as part of a control programme. Cortisol was higher in the boreal out-group than in our study population from the northern boreal forest.

5. Overall, our findings support the social and physiological consequences of human-caused mortality. Long-term implications of altered physiological responses should be considered in management and conservation strategies.

Key-words: boreal forest, *Canis lupus*, cortisol, grey wolves, hair analysis, human-caused mortality, northern Canada, progesterone, testosterone, tundra–taiga

Introduction

Human-caused mortality such as hunting and predator control has a multitude of effects on hunted populations (e.g. Coltman *et al.* 2003; Darimont *et al.* 2009). Despite documented population-level changes, only a few studies have examined the physiological effects of hunting, which reflect individuals' perceptions of and responses to environmental

conditions (Hofer & East 2012). For example, elevated stress hormones (e.g. cortisol) revealed that hunted individuals can be physiologically stressed by pursuit (Bateson & Bradshaw 1997) as well as by the disrupted social structure caused by hunting (Gobush, Mutayoba & Wasser 2008). Human activities and vehicular noise associated with hunting may also influence wildlife behaviour (Ciuti *et al.* 2012) and stress hormones (Creel *et al.* 2002).

Reproductive hormones (e.g. testosterone and progesterone) would provide additional insight into the effects of hunting on social structure and reproduction. Testosterone,

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which is integral to male reproductive activity, secondary sexual characteristics and behaviour, is also modulated by the social competitive environment and may be elevated when social conditions are unstable (Wingfield, Lynn & Soma 2001; Oliveira 2004). Notably, cortisol, testosterone and progesterone are elevated in females during pregnancy and can serve as indicators of population-level reproductive activity (Wasser *et al.* 1996; Foley, Papageorge & Wasser 2001). As with males, testosterone in females might influence or reflect the social environment (Albert, Jonik & Walsh 1992; Bryan *et al.* 2013b).

Hair, which integrates steroid hormones over several months to years, provides a valuable approach for investigating physiological responses to natural processes and potential long-term stressors (Meyer & Novak 2012). Unbound (free) steroids are thought to be incorporated into growing hair via the blood vessel that feeds the hair follicle and may also come from local synthesis of steroids in the follicle and/or from secretions of glands surrounding the hair follicle (Ito *et al.* 2005; Pragst & Balikova 2006; Keckeis *et al.* 2012). Accordingly, levels of hormones in hair have been correlated with measures in saliva and faeces, which are known to reflect circulating levels, in canids and other species (Davenport *et al.* 2006; Accorsi *et al.* 2008; Bennett & Hayssen 2010). Moreover, a number of recent studies have revealed that hormone levels in hair reflect meaningful biological and ecological patterns (e.g. Koren & Geffen 2009a; Macbeth *et al.* 2012; Bourbonnais *et al.* 2013; Bryan *et al.* 2013b; Malcolm *et al.* 2013).

Wolves (*Canis lupus*) are a suitable model for examining the physiological effects of human-caused mortality, which encompasses killing for recreational, commercial and predator control purposes. For millennia and across their Holarctic range, wolves have been targets of predator control strategies and hunting for commercial fur (Musiani & Paquet 2004). In North America, wolves are hunted at rates of 0–50% of the extant population annually, and occasionally up to 80–90% for short periods as part of intensive control programmes (Haber 1996; Fuller, Mech & Chochrane 2003). Though wolves respond to moderate population reduction through increased recruitment, exploitation affects behaviour, social group composition and genetic structure (Rausch 1967; Haber 1996; Jędrzejewski *et al.* 2005; Sidorovich *et al.* 2007; Rutledge *et al.* 2010, 2012). Wolves are particularly susceptible to social disruption from high mortality because their complex social structure affects many aspects of wolf population dynamics (Haber 1996). To date, no studies have examined the effects of human-caused mortality on wolf hormone levels, which could mediate or reflect changes in behaviour, reproductive activity and social structure. Moreover, long-term hormone levels in wolves, through measurements in hair, have not been previously documented.

Accordingly, our objective was to compare hormone levels in hair from wolves subject to different hunting pressures in two regions of northern Canada. Wolves were killed for fur and/or as part of government control pro-

grammes in the three study regions. The three areas differed in the hunting methods used, the proportion of the population likely removed and the social instability resulting from the removal of animals. Specifically, wolves in the tundra–taiga are thought to experience persistent, high levels of human-caused mortality and are often hunted by snowmobile using guns, whereas wolves in the boreal forest experience lower human-caused mortality and are killed mainly using traps and snares (Cluff & Murray 1995; Musiani & Paquet 2004; Cluff *et al.* 2010). On the basis of established links between high mortality, social instability and enhanced reproduction in wolves (Packard & Mech 1980, 1983; Packard, Mech & Seal 1983; Haber 1996), we predicted that tundra–taiga wolves would show higher levels of stress and increased reproductive effort through chronically increased hormone production (i.e. cortisol, testosterone and progesterone) compared with wolves in the boreal forest, which experience lower hunting pressures. To control for differences in habitat between these regions, we examined cortisol levels in an out-group of boreal forest wolves that is undergoing population reduction through a government control programme. Due to a lack of genetically confirmed sex data in the boreal out-group, we were unable to compare progesterone and testosterone levels with those in the other boreal forest group.

As part of our biological validation of this approach, we investigated the potentially confounding effect of hair colour on hormone levels in the wolf hair, because melanin content might play a role in steroid incorporation into hair (Pragst & Balikova 2006). Specifically, we predicted that dark hair would incorporate or retain more steroids due to its higher melanin content. Finally, we examined age structure from an out-group of heavily hunted tundra–taiga wolves for a literature comparison with other wolf populations subject to heavy hunting pressure.

Materials and methods

STUDY AREA AND SAMPLE COLLECTION

The wolf samples ($n = 152$) were collected from hunters as part of a previous study of wolves from Nunavut, the Northwest Territories, and Alberta, Canada (Fig. 1) (Musiani *et al.* 2007). The numbers of samples used in hormonal analyses from different regions are shown in Fig. 1. These wolves inhabit a contiguous landscape of coniferous boreal forest in the south extending to treeless arctic tundra in the north. The transition area between boreal forest and tundra is known as taiga, and serves as a boundary between morphologically and genetically distinct wolf populations (Musiani *et al.* 2007). Tundra–taiga wolves prey primarily on barren-ground caribou (*Rangifer tarandus*), which are seasonally available to wolves from late summer to winter (Parker 1973; Walton *et al.* 2001; Musiani *et al.* 2007). In the boreal forest, wolves prey on moose (*Alces alces*), elk (*Cervus elaphus*), caribou, bison (*Bison bison*) and deer (*Odocoileus* spp.) (Carbyn 1983; Carbyn & Trottier 1988; Urton & Hobson 2005).

Unlike wolf populations elsewhere in North America, wolves in the tundra–taiga and northern boreal forest regions that we studied experience low levels of human activity for most of the year.

However, hunting and trapping of wolves occur throughout the area in the winter months when fur is prime (Cluff *et al.* 2010; Webb, Allen & Merrill 2011). In the largely roadless tundra–taiga, hunting takes place when lakes are frozen so that areas where wolves and caribou over winter can be accessed by snowmobile (Cluff *et al.* 2010). Hunts in the tundra–taiga vary in intensity among years and locations but can be substantial as many wolf packs congregate near caribou herds in the winter (Cluff *et al.* 2010). Most snowmobile hunts are carried out by a few hunters each year. Trapping typically does not occur in the tundra–taiga because wolves are unlikely to frequent trails in the open habitat (Cluff *et al.* 2010). In contrast, wolves in the boreal forest are typically killed along established trap lines that are dispersed across the landscape with mortality rates that are relatively consistent over space and time (Webb, Allen & Merrill 2011).

We classified wolves as living in tundra–taiga or boreal forest regions according to the genetic analyses by Musiani *et al.* (2007). As part of their study, Musiani *et al.* (2007) classified coat colour of northern boreal and tundra–taiga wolves into light (white to near-white) or dark (grey to black). We used these categories to examine whether coat colour influenced hormone incorporation into hair. All wolf hair samples were collected during winter when the hair is not growing (Cluff *et al.* 2010). Wolves show an annual cycle of hair growth with moult occurring in early spring; therefore, new hair would start incorporating hormones in spring (reproductive season) and continue growing into the fall until it reaches its maximum length (Dumont & Reimchen 2002). Consequently, the samples we examined would encompass the latter part of gestation in female

wolves, and the summer season when pups are being reared. Sex of all individuals was determined genetically.

HAIR SAMPLES FROM AN OUT-GROUP OF HEAVILY HUNTED BOREAL FOREST WOLVES

To control for habitat effects between tundra–taiga and boreal forest wolves, we also examined cortisol levels in an out-group of heavily hunted wolves ($n = 30$) from the Little Smoky region of boreal forest in Alberta, Canada (Fig. 1). These samples were collected in winter between 2007 and 2008 as part of an ongoing predator control programme to protect an endangered population of mountain caribou (*R. tarandus caribou*) (Neufeld 2006; ASRA & ACA 2010). Wolves are removed by trapping, by aerial gunning from helicopters and by poisoning (Carbyn 2013). No genetic information on sex was available for these samples, which precluded our ability to compare reproductive hormones in these samples with those from the other two populations.

MEASUREMENT OF STEROIDS IN HAIR

The time between wolf collection and hair sampling varied. Hair samples were collected either from fresh carcasses or from thawed carcasses that had been frozen outside at temperatures below -20°C for up to 3 months. Tufts of hair (20–200 mg) attached to pieces of hide were cut from unknown body locations. Although body region may affect hair growth and hormone deposition

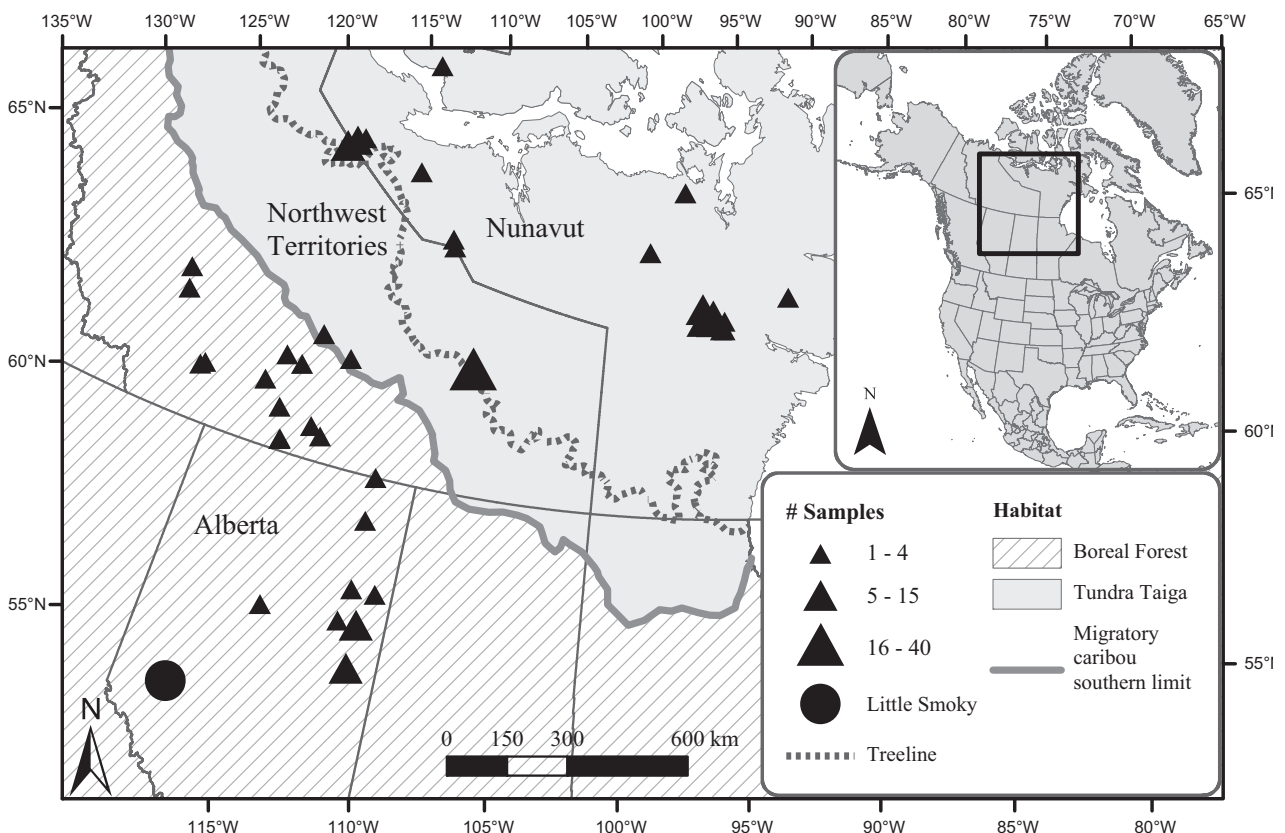


Fig. 1. Location of wolf hair samples from tundra–taiga and boreal forest habitats in Nunavut, the Northwest Territories, and Alberta, Canada. Samples from the Little Smoky wolf pack ($n = 30$) represent a heavily hunted out-group in the boreal forest. The size of the symbols is proportional to the number of samples collected. We classified wolves as tundra–taiga if they were collected north of the southern limit of caribou migration (solid grey line), following Musiani *et al.* (2007).

(Macbeth *et al.* 2010; Terwissen, Mastromonaco & Murray 2013), a directional bias among populations is unlikely. Following collections, hair samples were dried thoroughly and stored at room temperature in paper envelopes for up to 13 years before hormonal assays (Bortolotti *et al.* 2009; Macbeth *et al.* 2010). Hair was cut from the hide with scissors as closely as possible to the root before hormonal analyses. Our protocol for extracting steroids from hair followed a procedure that has been previously described and validated in dogs (*Canis familiaris*) (Bryan *et al.* 2013a) and bears (*Ursus* spp.) (Bryan *et al.* 2013b). In brief, we washed hair samples twice with water and twice with isopropanol for 3 min per wash while rotating the samples at 130 rpm. Samples were thoroughly dried between the water and isopropanol washes. Next, we ground the hair to a fine powder (c. 5 min/50 mg of hair at 65 Hz, Mixer Mill 200; Retsch, Haan, Germany), weighed 30 mg of powder into a glass scintillation vial and added 50 µL of methanol (Omnisolv; VWR, Mississauga, ON, Canada) per mg of hair powder. To extract steroids, we sonicated the samples for 30 min and rotated them at 160 rpm in an incubator for 18 h at 50 °C. After centrifuging, we aliquotted the supernatant into separate tubes for progesterone (100 µL), testosterone (50 µL) and cortisol (1000 µL) assays and evaporated the methanol under a stream of nitrogen. We used commercial kits designed for saliva, to measure cortisol, progesterone and testosterone in hair extracts (Salimetrics, Philadelphia, PA, USA). To reduce interassay bias, we randomly assigned samples to plates.

We assessed parallelism for each assay by comparing a serially diluted extract of hair pooled from 10 wolves (five males and five females) with assay standards. Analysis of covariance tests comparing the slopes of the regression lines for standards and serially diluted hair samples revealed that diluted wolf hair extracts were parallel with assay standards ($P = 0.221$ for cortisol, $P = 0.943$ for testosterone and $P = 0.962$ for progesterone). Other validation parameters for the three assays, including inter- and intraspecific coefficients of variation, recovery and repeatability, have been previously reported (Bryan *et al.* 2013a,b). All coefficients of variation were <15%, with the exception of the low control for progesterone, which had a mean interassay coefficient of variation of 25% on the basis of 5 plates (runs). None of the wolf samples had a concentration in the range of the low control, so this variability would not have affected the results. Cross-reactivity of each assay to a number of non-target compounds was provided with the immunoassay kits. Specifically, the cortisol assay was tested by the kit manufacturer (Salimetrics) for cross-reactivity to aldosterone (0.03%), betamethasone (1.6%), corticosterone (0.94%), cortisone (0.98%), danazol (0.01%), 11-deoxycorticosterone (0.26%), 11-deoxycortisol (11.4%), dexamethasone (0.04%), estriol (0.01%), estrone (0.007%), flumethasone (0.017%), methotrexate (0.004%), methylprednisolone (12%), prednisolone (76%), prednisone (2.3%), pregnenolone (0.02%), progesterone (0.02%), tetrahydrocortisol (0.34%) and triamcinolone (0.13%). For the testosterone assay (Salimetrics), the reported cross-reactivity was aldosterone (<0.004%), androstenedione (1.157%), corticosterone (<0.004%), cortisol (<0.004%), cortisone (<0.004%), 11-deoxycortisol (<0.004%), 21-deoxycortisol (0.004%), DHEA (<0.004%), dianabol (0.489%), dihydrotestosterone (36.4%), epitestosterone (0.165%), 11-hydroxytestosterone (1.90%), 19-nortestosterone (21.02%), epitestosterone (0.165%), estradiol (0.025%), estriol (0.012%), estrone (0.005%), progesterone (0.005%), 17 α -hydroxyprogesterone (<0.004%) and transferrin (<0.004%). For the Salimetrics progesterone kit, the reported cross-reactivity was aldosterone (<0.004%), corticosterone (0.1924%), cortisol (<0.004%), cortisone (0.0106%), 11-deoxycortisol (0.0195%), 21-deoxycortisol (0.0082%), dexamethasone (0.0014%), DHEA (<0.004%), estradiol (<0.004%), estriol (<0.004%), estrone (<0.004%), 17 α -hydroxyprogesterone (0.0723%), prednisolone (0.0021%), prednisone (0.0038%), testosterone (<0.004%), transferrin (<0.004%) and triamcinolone (<0.004%).

AGE DETERMINATION IN A HEAVILY HUNTED OUT-GROUP OF TUNDRA-TAIGA WOLVES

To examine the extent to which hunting influences age structure among tundra-taiga wolves, we collected the teeth from wolves that had been killed between 1970 and 2000 ($n = 144$) in a 13 000 km² area surrounding Rennie Lake, Northwest Territories, Canada. Wolves in the Rennie Lake area have been subject to persistently high annual mortality for several decades and are therefore exemplary of the hunts that occur in the tundra-taiga (Cluff *et al.* 2010). In order of preference depending on which teeth were present, we selected the first molar (M1), followed by the second molar (M2), canines, incisors and premolars. The teeth were sent to a commercial laboratory for ageing using cementum annuli (Mattson's Laboratory, Milltown, MT, USA). None of the wolves with tooth samples had corresponding hair for hormonal analyses. However, the tooth samples were collected from wolves killed over several years from an area with similar habitat characteristics to those where hair samples were also collected. Therefore, we assumed tooth samples would provide age data representative of wolf populations hunted in the tundra-taiga. Unfortunately, comparable data on age were not available for wolves from the boreal forest. Therefore, we compared age structure in tundra-taiga wolves with similar studies from the literature to gain insight into potential effects of high levels of human-caused mortality on wolf age structure.

STATISTICAL ANALYSIS OF HORMONAL DATA

Progesterone, cortisol and testosterone measurements in wolf hair had skewness values of 0.9, 1.0 and 2.4, respectively, indicating that the distributions were right-skewed (i.e. towards higher values) relative to a normal distribution with skewness of 0. A natural log transformation improved the normality of progesterone and cortisol measurements; however, a stronger, negative reciprocal transformation ($-1/x$) was required for testosterone. Model assumptions of equality of variances (Levene's tests) and normality of residuals (Kolmogorov-Smirnov tests) were met. We used multi-way analysis of variance (ANOVA) to compare differences in cortisol and testosterone between sexes, habitat types and coat colour categories. We included an interaction term between sex and habitat to determine whether any differences between sexes were consistent between the two habitats. Among females, we examined differences in progesterone between habitat types and hair colour using an ANOVA. No data were available on sex of the Little Smoky samples; therefore, we compared cortisol across both sexes among the three populations using an ANOVA with Welch's approximation for unequal variances. To examine pairwise contrasts, we used Welch's *t*-tests and applied Holm's correction factor on the resulting *P*-values to account for the number of comparisons. We conducted all analyses using R statistical software with $\alpha = 0.05$ (R Development Core Team 2011).

Results

HORMONES IN HAIR FROM WOLF POPULATIONS IN THE TUNDRA-TAIGA AND BOREAL FOREST

We found differences by sex and area in stress and reproductive hormones of wolves from the tundra-taiga and the northern boreal forest (Fig. 2; Table 1). Female wolves from the tundra-taiga had higher progesterone ($F_{1,66} = 10.73$, $P = 0.002$) compared with wolves in the boreal forest (Fig. 2a). Testosterone levels were higher in

tundra–taiga wolves of both sexes ($F_{1,143} = 6.12$, $P = 0.014$). Males had higher testosterone than females ($F_{1,143} = 7.79$, $P = 0.005$) and the trend was consistent across habitat types ($F_{1,143} = 0.107$, $P = 0.744$; Fig. 2b). Tundra–taiga wolves had higher cortisol, compared with boreal forest wolves ($F_{1,143} = 5.24$, $P = 0.023$; Fig. 2c). Cortisol did not differ between sexes ($F_{1,143} = 2.98$, $P = 0.086$). Although cortisol appeared similar among female wolves in the two habitats, there was no evidence of an interaction between sex and habitat ($F_{1,143} = 2.27$, $P = 0.134$; Fig. 2c). Finally, there was no effect of hair colour on cortisol ($F_{1,143} = 0.52$, $P = 0.470$), testosterone ($F_{1,143} = 0.04$, $P = 0.744$) or progesterone ($F_{1,66} = 0.67$, $P = 0.416$).

HORMONES IN AN OUT-GROUP OF BOREAL FOREST WOLVES FROM A POPULATION CONTROL PROGRAMME

With sexes combined, cortisol differed among wolves from the tundra–taiga, northern boreal forest and the boreal, Little Smoky region with population control management ($F_{2,57} = 3.68$, $P = 0.031$; Fig. 3). Pairwise comparisons with adjusted P -values revealed that wolves from the Little Smoky region had higher cortisol compared with wolves in the northern boreal forest ($t_{52} = 2.61$, $P_{\text{adj}} = 0.023$; Fig. 3). Little Smoky wolves had similar cortisol to wolves in the tundra–taiga ($t_{34} = 1.66$, $P_{\text{adj}} = 0.106$; Fig. 3).

AGE STRUCTURE FROM AN OUT-GROUP OF WOLVES IN THE TUNDRA–TAIGA

In samples from wolves in the tundra–taiga ($n = 144$), the average age was 2 years (range: 0–9 years). Pups (<1 year) represented 39% of the population and only 11% of individuals were more than 5 years in age (Table 2).

Discussion

Physiological responses are adaptive mechanisms by which organisms respond to complex interactions among individual, social and environmental conditions. As predicted, wolves from heavily hunted populations had higher stress and reproductive hormone levels which probably reflect a number of environmental conditions including human-caused mortality. Although we were not able to confirm successful pregnancies in individual wolves, the higher progesterone we detected in female tundra–taiga wolves compared with forest wolves is consistent with increased reproductive activity because of social disruption as well as established numeric responses of wolves to high mortality rates (Fuller, Mech & Chochrane 2003; Adams *et al.* 2008). The hair samples most likely reflect progesterone levels in the spring before collection when the hair was grown. This period corresponds with the latter stages of pregnancy or pseudopregnancy, both of which might occur more frequently among female wolves from packs where social structure is disrupted. Reproduction in wolves is

regulated through well-established relationships among pack members (Packard & Mech 1980, 1983; Packard, Mech & Seal 1983). In most stable social groups, wolves have only one litter per year (Harrington *et al.* 1982; Paquet, Bragdon & McCusker 1982; Packard, Mech & Seal 1983), even though non-breeding individuals show normal reproductive cycles and are capable of reproducing (Packard *et al.* 1985). When social structure is disrupted, multiple litters per social group become more common, in part because dominant individuals can no longer prevent subordinates from breeding (Packard *et al.* 1985; Haber 1996). Notably, large litters with multiple lactating females have been observed in the tundra–taiga wolf population that we studied (Cluff *et al.* 2003; Frame *et al.* 2004).

In males, higher testosterone among tundra–taiga wolves is unlikely to reflect higher reproductive activity since hair does not grow – and therefore is unlikely to incorporate steroid hormones – during the breeding season, which occurs in winter. Instead, we propose that higher cortisol and testosterone levels in tundra–taiga wolves are consistent with social instability caused by an increased frequency of interindividual interactions that have unpredictable outcomes (Goymann *et al.* 2001; DeVries, Glasper & Detillion 2003; Creel *et al.* 2013). Notably, culling has been previously shown to disrupt social structure, resulting in increased dispersal and disease transmission (McDonald *et al.* 2008). Hunting can also decrease pack size, which results in altered predation patterns, increased time spent defending kill sites from scavengers and may lead to increased conflict with humans and livestock (Hayes *et al.* 2000; Wydeven *et al.* 2004; Zimmermann 2014). Physiological changes in response to disrupted social structure, predation patterns and/or pack size would likely be adaptive. In particular, cortisol prepares individuals to cope with social conflict in a number of ways, including by mobilizing energy stores and increasing muscle tone (Sapolsky 1993). Similarly, elevated testosterone may help individuals cope with social challenges by

Table 1. Median (M), range (R) and sample size (n) for progesterone, testosterone and cortisol in wolf hair samples collected from hunted wolves in the tundra–taiga and northern boreal forest of Canada

	Northern forest			Tundra–taiga		
	M	R	n	M	R	n
Progesterone (pg mg ⁻¹)						
Females	19.9	13.2–34.8	23	27.0	12.8–53.3	46
Testosterone (pg mg ⁻¹)						
Females	4.9	3.3–10.8	24	5.0	3.5–9.3	48
Males	5.5	3.3–9.6	21	5.3	3.9–15.1	55
Cortisol (pg mg ⁻¹)						
Females	14.6	7.6–34.0	24	17.3	9.95–32.2	48
Males	12.3	4.8–26.8	21	15.8	8.91–40.4	55

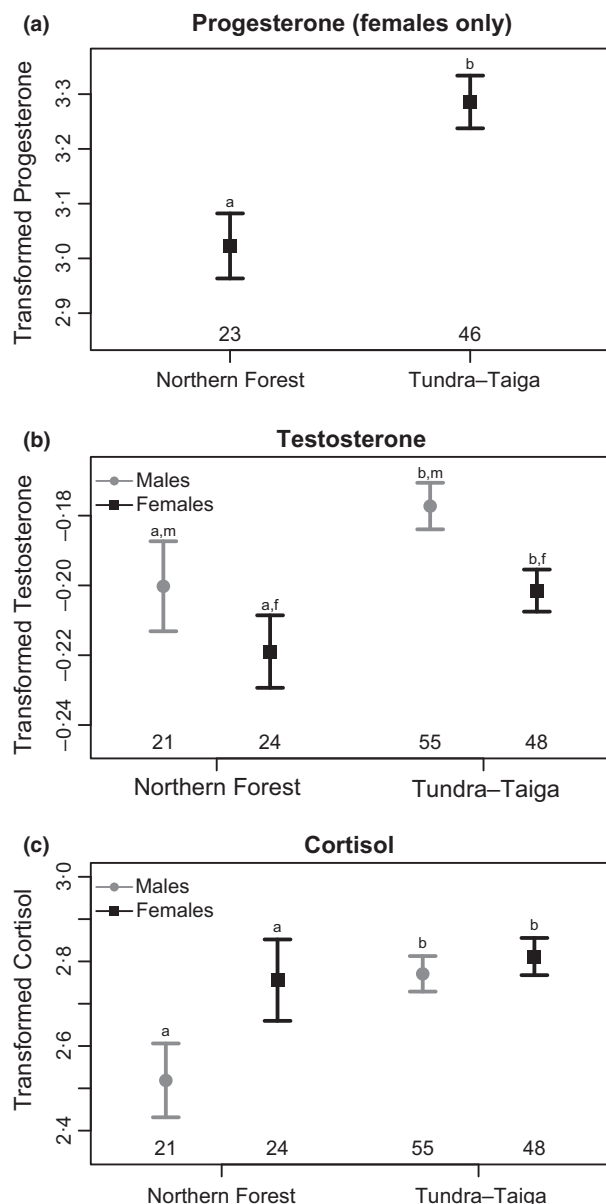


Fig. 2. Levels of hair progesterone (females only, a), testosterone (b) and cortisol (c) for female and male wolves from the tundra-taiga and northern forest regions of Canada. Error bars represent standard error. To improve normality of residuals, cortisol and progesterone data were transformed (see Statistical Analysis of Hormonal Data). The sample sizes are shown below the error bars. Symbols 'a, b' denote differences between populations and 'm, f' denote differences between sexes.

affecting behaviour, muscle mass and other traits related with social interactions in fitness-enhancing situations (Wingfield, Lynn & Soma 2001; Oliveira 2004).

Ecological or genetic differences between tundra-taiga and boreal forest wolves could contribute to the hormonal trends we observed. In particular, patterns in territoriality differ between populations; tundra-taiga wolves spend most of the year following caribou and maintain territories only during the summer breeding season (Parker 1973; Walton *et al.* 2001; Musiani *et al.* 2007). Aggregations of

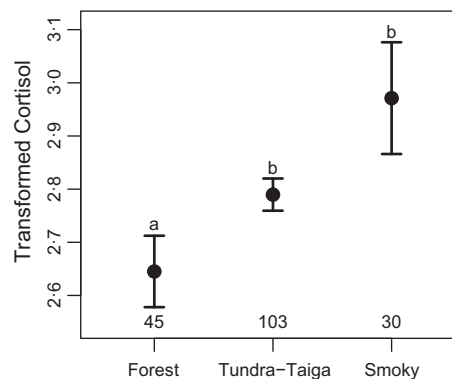


Fig. 3. Cortisol in a heavily hunted out-group of wolves of both sexes from the Little Smoky Caribou Range in the central boreal forest, and for comparison, in wolves from the northern boreal forest and tundra-taiga regions also pooling sexes. Error bars represent standard error. The sample sizes are shown below the error bars. Symbols 'a, b' denote differences among populations.

Table 2. Age structure in an out-group of hunted wolves ($n = 144$) from the tundra-taiga

Age	Number	Proportion
0–1	56	0.39
1–2	24	0.17
2–3	23	0.16
3–4	16	0.11
4–5	9	0.06
5–6	9	0.06
6–7	1	0.01
7–8	3	0.02
8–9	2	0.01
9–10	1	0.01

tundra-taiga wolves near caribou herds when caribou return in late summer could provoke intergroup interactions, and consequently, higher testosterone levels. In contrast with tundra-taiga wolves, wolves in the boreal forest have established, year-round territories where social conditions should be relatively stable (Walton *et al.* 2001; Musiani *et al.* 2007). Thus, social and ecological differences between populations could contribute to higher testosterone in tundra-taiga wolves.

In addition, elevated cortisol in tundra-taiga wolves could reflect an adaptive response to prolonged food shortages during summer, when caribou migrate beyond wolf den sites and wolves must travel or rely on alternative prey (Frame *et al.* 2004). Similarly, lactating female spotted hyenas (*Crocuta crocuta*) had elevated faecal glucocorticoids in response to long-distance foraging movements combined with social stress during periods of low prey availability (Goymann *et al.* 2001). In contrast with hyenas that do not provide communal care for pups, all members of wolf packs that share provisioning and parental care activities might show elevated cortisol when food availability near the den site is low. Indeed, cortisol is thought to

be an adaptive response to prolonged food shortages in wolves and other species. Higher circulating cortisol stimulates the mobilization of energy fat stores and may also affect cognitive processes and behaviour relating to foraging (Pravosudov 2003; Behie, Pavelka & Chapman 2010). However, we found that wolves from the Little Smoky wolf population, which inhabit the boreal forest but have experienced severe depopulation as part of a wolf control programme – had higher hair cortisol than wolves in similar boreal forest habitat where human-caused mortality is substantially lower. This finding provides evidence that elevated hair cortisol reflects disruption caused by high mortality rates in addition to or in combination with an effect of habitat.

The average age of tundra–taiga wolves in our study (2.0 years) was intermediate to the average ages of 1.5 and 2.8 years reported in a comparable study during periods of high and low hunting pressure, respectively (Sidorovich *et al.* 2007). Similarly, the proportion of tundra–taiga wolves <1 year old in our study (39%) was intermediate to proportions of 55% and 34% reported during periods of high and low hunting (Sidorovich *et al.* 2007). This comparison suggests that tundra–taiga wolves could have a skewed age structure and higher proportion of juveniles relative to populations with lower hunting pressure. However, other studies suggest that wolves have a high population turnover in the absence of hunting, making it difficult to confirm whether age structure provides a useful reflection of social instability (Rausch 1967; Mech 2006). Therefore, assessments of social structure should include information on the persistence of socially important and reproductive individuals within packs (e.g. the dominant and breeding pairs; Borg *et al.* 2014) as the large population turnover in unprotected populations could have fundamentally different ecological and evolutionary implications than that in protected populations (Rutledge *et al.* 2010, 2012).

In addition to more information on age and social structure, future studies should address the effects of different types of hunting on wolf physiology and possible sampling bias. Specifically, hunters using snowmobiles on the relatively open tundra–taiga might kill a large proportion of a pack indiscriminate of age, whereas trappers in the boreal forest might remove a smaller proportion of each pack and could be more likely to catch a certain cohort (e.g. juveniles) (Webb, Allen & Merrill 2011). However, government control programmes, such as the one applied to Little Smoky wolves, target entire family groups. These different approaches could differentially affect wolf social structure and behaviour at the pack and population levels and might lead to sampling bias.

The auditory, olfactory or physical invasiveness of different hunting methods could also influence wolves' physiological responses. Specifically, hunted or radiocollared wolf populations show notably different behavioural responses to human presence, such as aircraft, compared with wolf populations surveyed, but not killed or captured,

from aircraft (P. C. Paquet, pers. obs.). Aerial gunning combined with higher year-round use of vehicles that wolves might associate with hunting could contribute to higher cortisol levels in the Little Smoky boreal out-group compared with other boreal wolves. Although wolves may show a stress response to snowmobile activity (Creel *et al.* 2002), this possibility is unlikely to explain elevated stress and reproductive hormones in hair of tundra–taiga wolves because snowmobile use occurs mostly during a short (3-month) time period by relatively few (5–12) hunters and when hair is not growing (Cluff *et al.* 2010).

A better understanding of hair growth and hormone deposition into hair would also help in making interpretations among populations. Steroid hormones are thought to accumulate gradually as the hair grows, providing a long-term record of endocrine activity (e.g. Kirschbaum *et al.* 2009; Ashley *et al.* 2011; Malcolm *et al.* 2013; Terwissen, Mastromonaco & Murray 2013). However, shorter term changes might occur via glands surrounding the hair follicle that secrete substances containing steroid hormones and/or from local synthesis of steroids in the hair follicle (Keckeis *et al.* 2012; Russell *et al.* 2014). Washing the hair samples is thought to minimize the effects of glandular and other external sources of steroid hormones on hair (Davenport *et al.* 2006; Macbeth *et al.* 2010). Accordingly, as with other studies of species with seasonal patterns in hair growth (e.g. Bryan *et al.* 2013b), we assumed that the hormones in wolf hair reflected endocrine activity and associated life-history events during hair growth. Although linking hair samples to a specific time period is more challenging in species with non-seasonal or unknown moult patterns, a number of studies have found that hair hormone levels reflect chronic, baseline levels relating to interesting biological and ecological patterns (Koren *et al.* 2002; Koren, Mokady & Geffen 2006; Koren & Geffen 2009a,b; Finkler & Terkel 2010; Bryan *et al.* 2013a; Galuppi *et al.* 2013). Ultimately, additional validations using carefully designed challenge experiments and longitudinal sampling will facilitate interpretation of steroid hormone measurements in mammals with both seasonal and non-seasonal moult patterns.

Different patterns in hair growth and associated hormone deposition into hair might have contributed to the trends we observed between regions. For example, tundra–taiga wolves might have different baseline hormone levels or adaptations for living in the arctic that could affect their patterns of hair growth. Similarly, the extent to which wolves are physiologically adapted to the local climate is unknown and consequently, colder temperatures and more dramatic temperature fluctuations might contribute to elevated cortisol levels among tundra–taiga wolves. Notably, differences in climate are unlikely to explain our results from Little Smoky wolves in the boreal forest. The timing of sampling also differed between regions. We assumed, however, that this would not affect our results because wolf hair does not grow over winter. Further studies of progesterone in hair are required to confirm increased

reproductive activity in the heavily hunted tundra–taiga wolves. Specifically, progesterone does not necessarily indicate successful pregnancy in wolves because pseudopregnant and pregnant females have similar endocrine profiles although the former is not, in fact, carrying foetuses (Seal *et al.* 1987; Kreeger 2003). In future studies, comparing the proportion of pregnant and pseudopregnant females in wolf packs under stable and unstable social conditions will be important (Stoops, MacKinnon & Roth 2012).

Though further studies will generate additional insights, our data provide the first baseline on chronic levels of stress and reproductive hormones in wolf hair. Our results of higher testosterone in hair of males and similar cortisol in hair of both sexes are consistent with studies in urine and serum (Seal & Mech 1983; Gadbois 2002), which reflect very recent (minutes) levels of circulating hormones. Notably, we did not detect a difference between wolves with white and black coat colour for any of the hormones, which is consistent with studies in humans (e.g. Sauvé *et al.* 2007) but not in other species, including dogs (Bennett & Hayssen 2010). Moreover, the cortisol concentrations we measured in wolf hair (median: 15.4 pg mg⁻¹; range: 4.8–40.4 pg mg⁻¹; $n = 148$) were similar to values in dogs of mixed age, breed, sex and coat colour measured using the same extraction and immunoassay procedure (median: 11.8 pg mg⁻¹; range: 3.7–19.7 pg mg⁻¹; $n = 7$) (Bryan *et al.* 2013a). Thus, hair cortisol levels appear to be similar across these two canid species.

In conclusion, the combination of elevated cortisol and sex steroids we observed in tundra–taiga wolves are probably explained by interacting effects of hunting pressure, habitat and/or sampling. Though we were not able to partition the relative importance of these factors, elevated cortisol in the heavily hunted boreal out-group suggests that hunting pressure at least contributes to the differences we observed. The potential physiological effects of substantial, human-caused mortality suggest that hunting could be causing changes in reproductive structure and breeding strategy, as well as imposing chronic stress. Though increased reproduction might be viewed as a positive response of wolves to population reductions, the implications on lifetime reproductive output and generational survival of offspring as compared with undisturbed populations are unknown. However, a predicted outcome of such population disturbances is the loss of genetic diversity that can lead to a decrease in individual fitness and evolutionary potential, as well as an increased risk of population extinction (Frankham, D. & A. 2002; Leonard, Vilà & Wayne 2005). Indeed, elevated stress and reproductive hormones in hair or feathers have been associated negatively with fitness (Koren *et al.* 2011) and proxies of fitness (Macbeth *et al.* 2012; Bryan *et al.* 2013b). Moreover, chronic stress may have evolutionary consequences for wolf populations via epigenetic, intergenerational changes (McGowan & Szyf 2010; Cao-Lei *et al.* 2014). Ultimately, our findings highlight the importance of considering factors other than population numbers when set-

ting management objectives (Haber 1996; Paquet & Darimont 2010; Borg *et al.* 2014). Furthermore, we add to a growing body of literature that hormonal measures in hair are a valuable tool for monitoring and informing conservation strategies.

Acknowledgements

Samples were collected and analysed under protocols approved by the Animal Care Committee at the University of Calgary (Musiani B111R-17, Smits B110R-01). We are grateful to the following people for help with logistics and sample collection: L. Adam, G. Bihun, M. Campbell, R. Case, D. Cluff, P. Frame, M. Hebblewhite, D. Hervieux, R. Mulders, J. Novembre, K. Smith, D. Stepnisky and L. R. Walton. For assistance with sample and data analysis, we thank L. Bond, A. De Sousa, R. Invik and B. Weckworth. This work was funded by the National Sciences and Engineering Research Council of Canada Discovery Grants to JES (RGPIN-22876-20), KEWE (RGPIN-106386-2008) and MM (RGPIN-327065-2011), the World Wildlife Fund (Canada), the Canadian Association of Petroleum Producers and the University of Calgary Faculty of Veterinary Medicine (UCVM). HMB was supported by an NSERC industrial post-graduate scholarship, the University of Calgary Queen Elizabeth II Scholarship Program, the UCVM Department of Ecosystem and Public Health Studentship Program, and the UCVM. LK was supported by a UCVM Post-doctoral Fellowship. A draft version of this paper was presented in the doctoral thesis of HMB. For current postdoctoral funding, HMB recognizes the Hakai Program (hakai.org) and the Tula Foundation (tula.org). We are grateful for helpful comments provided by two anonymous reviewers and an associate editor, which substantially improved the manuscript.

Data accessibility

Data analysed for this manuscript are publicly available in the Dryad repository at <http://dx.doi.org/10.5061/dryad.5fp5m> (Bryan *et al.* 2014).

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Received 9 February 2014; accepted 8 October 2014

Handling Editor: Jennifer Grindstaff