PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)

Folia Biologica (Kraków), vol. 63 (2015), No 4
doi:10.3409/fb63_4.229

Plasma Glucocorticoids and ACTH Levels During Different Periods of Activity in the European Beaver (*Castor fiber* L.)*

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Accepted September 10, 2015

CZERWIŃSKA J., CHOJNOWSKA K., KAMIŃSKI T., BOGACKA I., PANASIEWICZ G., SMOLIŃSKA N., KAMIŃSKA B. 2015. Plasma glucocorticoids and ACTH levels during different periods of activity in the European beaver (*Castor fiber* L.). Folia Biologica (Kraków) **63**: 229-234.

Glucocorticoids (GCs) and adrenocorticotropic hormone (ACTH) are major components of the classic endocrine stress response. Free-living vertebrates are characterized by circannual changes in the baseline and/or stress-induced secretion of GCs and ACTH. In mammalian species, GC and ACTH levels vary seasonally but there is no consensus to the season in which animals have elevated GC and ACTH levels. The aim of our study was to determine, for the first time, the type and amount of glucocorticoids produced in free-living beaver (*Castor fiber* L.) – the largest rodent in Eurasia, and to find out whether stress-induced plasma GC and ACTH levels show seasonal variations. Blood samples were obtained from animals under general anesthesia in April (pregnancy in females), July (offspring rearing) and November (preparing for the winter). The adrenals of beavers produce both cortisol and corticosterone, and plasma cortisol levels were higher than corticosterone. In the current experiment, plasma cortisol concentrations in beavers were affected by the season. The highest stress-associated cortisol levels were noted in males in July during offspring rearing. Corticosterone and ACTH concentrations in beavers remained generally constant, regardless of the season and sex. In conclusion, seasonal changes were observed only in relation to stress-induced plasma cortisol levels in the beaver.

Key words: Glucocorticoids, ACTH, seasonal changes, beaver.

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Glucocorticoids (GCs) and adrenocorticotropic hormone (ACTH), the main hormones that regulate the production and release of GCs, are major components of the classic endocrine stress response (ROMERO 2002; ROMERO *et al.* 2008; SAPOLSKY *et al.* 2000). In a group of small mammals (e.g. rats, voles, rabbits), corticosterone is the primary GC (AMIRAT *et al.* 1980; GALEA & MCEWEN 1999; KRISHNA *et al.* 1998). In some species (e.g. hamsters, guinea pigs, arctic ground squirrels, degu), both GCs (cortisol and corticosterone) are produced in different proportions (BOONSTRA *et al.* 2001; KENAGY *et al.* 1999; KOLLACK-WALKER *et al.* 1999; PROVENCHER *et al.* 1992); cortisol generally has much greater biological activity than corticosterone (SHERIFF *et al.* 2011). Many free-living vertebrate species are characterized by circannual changes in the baseline and/or stress-induced secretion of GCs (ROMERO 2002). This helps animals respond to environmental threats and protects them through inducing a variety of behavioral and physiological changes (READER & KRAMER 2005). It has been suggested that plasma GC levels can be modified by environmental factors (e.g. photoperiod, temperature) and changes in the animal's physiological state. They can be also influenced by sex and stage of development (ROMERO *et al.* 2008). In

*Supported by the National Science Centre (project no: 2012/07/B/NZ9/01335), the Ministry of Higher Education (project no. 528/0206/882) and individual scholarships from PhD students programs (CIiTT/RIM WiM/2014/59 and POKL.08.02.02-20-002/12).

mammals, GC concentration varies seasonally but there is no consensus as to the season in which animals have elevated GC levels (for review see: ROMERO 2002). Lack of a common pattern of annual changes in different mammalian species is probably connected with evolutionary consequence in the risk of being exposed to stressors (CAHILL *et al.* 2013; ROMERO 2002; SAPOLSKY *et al.* 2000).

Seasonal rhythms of adrenal gland activity may be related to seasonal changes in the secretion of ACTH. To date, few studies have investigated changes in ACTH levels, including their seasonal patterns in mammals, and those that have been conducted deal mainly with laboratory and domesticated animals (AMIRAT & BRUDIEUX 1993; COPAS & DURHAM 2012; OTSUKA *et al.* 2012).

The Eurasian beaver (*Castor fiber* L.) is the largest free-living rodent in Eurasia. Beavers show seasonal patterns of reproduction. Peak reproductive activity is observed towards the end of winter, pregnancy lasts 105-107 days, and the young are born in May and June. Beavers do not hibernate, and therefore before the winter they accumulate subcutaneous fat deposits (NOLET & ROSELL 1998; ŻUROWSKI 1992). In contrast to many other rodent species, the physiology of the Eurasian beaver remains poorly investigated. The beaver is a protected species in most member states of the European Union (Convention on International Trade in Endangered Species of Wild Fauna and Flora and the Bern Convention on the Conservation of European Wildlife and Natural Habitats).

Seasonal variations in adrenal function, including the type, concentration and proportion of secreted GCs and ACTH, remain completely unexplored in the beaver, although they have been documented in other seasonal mammals. The study of stress-induced GC and/or ACTH levels were conducted in domestic animals and laboratory and free-living rodents (AMIRAT *et al.* 1980; CORDERO *et al*. 2012; DONALDSON *et al*. 2005; FUNK *et al*. 2011; GALEA & MCEWEN 1999; LE GOASCOGNE *et al.* 1991, MONAMY 1995; ROMERO *et al.* 2008). Therefore, we hypothesize that both stress-induced plasma GC (cortisol and corticosterone) and ACTH levels exhibit seasonal variation.

Material and Methods

Animals

Animals were taken from the north-eastern region of Poland (district Warmia and Mazury) with approval of the Regional Directorate of Environmental Protection in Olsztyn, Poland (ministerial approval: RDOS-28-OOP-6631-0007-638/09/10/pj). All studies were conducted in accordance with ethical standards of the institutional Animal Ethical Committees (SGGW/11/2010 and UWM/111/2011/DTN). Because of the species protection, the number of individuals sampled is relatively small, and they were obtained from different habitats. They were caught over a period of two years (2011/2012) by the same group of qualified staff. Beavers were captured at night. After capture, they were placed in cages and transported (2-3 h) to the laboratory of the Research Station of Ecological Agriculture and Preservation Animal Breeding of the Polish Academy of Sciences in Popielno. Immediately after delivery, each beaver was weighed in the cage, and the weight of the empty cage was subtracted. Blood samples were obtained from mature animals under general anesthesia, after two anesthetic drug injections of xylazine (Sedazin®, Biovet Pulawy, Poland, 3 mg/kg of BW) and ketamine (Bioketan, Vetoquinol Biowet, Poland, 15 mg/kg of BW) at the same time of day (from 8 a.m. to 12 p.m.) in April – 'gestation', males living with females (8 males and 5 pregnant females), July – 'post-breeding', the end of lactation and raising offspring (4 males and 6 females) and November – 'pre-breeding', sexual quiescence and preparing for the winter (6 males and 5 females). Samples were not taken from January to March (mating season) because of the difficulties of hunting beavers in winter (animals remain in their lodges, often under the ice). The chemicals used were chosen so as not to affect the biological condition of other tissues, which were destined for other analyses. At the end of the study, beavers were sacrificed by decapitation under full anesthesia. Average body weight (BW) of the captured animals was 18.4 ± 0.76 kg for the males and 19.8 ± 0.64 kg for the females. Blood samples were collected (2-3 h after capture) from the carotid artery into heparinized tubes, centrifuged (2500xg, 10 min, 4°C) and the obtained plasma was stored at -70°C, to avoid loss of bioactivity and contamination, until the measurements.

Plasma GC and ACTH concentrations

Total cortisol and corticosterone concentrations were determined in plasma samples (20 µl), after ethyl acetate extraction, using a radioimmunoassay method as described by KOTWICA *et. al* (2004). The mean extraction efficiency was more than 90 % for both glucocorticoids. The specificity of the antibodies against cortisol (SO/F/2) and corticosterone (SO/B/6) was reported by SZAFRAŃSKA *et al*. (2002). Validity of the assays was confirmed by parallelism between the standard curves and series of dilutions of randomly chosen plasma samples. Intra – and inter – assay coefficients of variation were less than 7% and the sensitivity of both assays was 0.015 ng/probe. Determination of ACTH levels was performed using an enzyme-linked immunosorbent assay. Due to a lack of specific beaver kits, the available commercial kit (Cusabio, China), with a wide detection range (6.25-100 pg/mL) was applied. The standard curve was parallel to the curve prepared from serially diluted blood plasma of beavers. The intra-assay coefficient of variation was less than 5%. Inter-assay factors of variation were not calculated (all analyses were done in one assay). The sensitivity of the assay was 3.12 pg/mL.

Statistical analysis

All data (ng/mL or pg/mL; mean±SEM) were analysed by two-way ANOVA followed by the Duncan *post hoc* test. Analyses were performed using Statistica software (StatSoft Inc., Tulsa, OK, USA). The level of significance was set at $P<0.05$ for all analyses.

Results

Glucocorticoids in beavers

Our findings indicate that the adrenal glands of beavers (n=34) produce both cortisol and corticosterone (Fig. 1) and cortisol is the main GC (F=44.12, df=1, P.00001). No significant differences between males and females in cortisol or corticosterone levels were observed (F=1.18, df=1, P=0.28). Both in males (n=18) and females (n=16), mean plasma cortisol levels (from all samples during three seasons) were higher ($P<0.05$) than corticosterone levels (23.9±2.56 ng/mL vs. 9.9±0.87 ng/mL and 20.5±2.3 ng/mL vs. 9.2±1.12 ng/mL, respectively).

Sex-related and seasonal changes in plasma cortisol, corticosterone and ACTH levels in beavers

Seasonal (F=3.70, df=2, P=0.04), but not sex-dependent (F=3.24, df=1, P=0.08) changes in plasma cortisol levels were observed. The highest ($P<0.05$) cortisol concentrations (Fig. 2A) were noted in the males (n=4) in July (37.6±4.06 ng/mL). Neither sex- (F=0.29, df=1, P=0.59) nor season-related (F=2.66, df=2, P=0.09) variation in plasma corticosterone levels were observed (Fig. 2B). However, corticosterone levels tended to be higher (P=0.051) in males (n=6) in November than in males (n=8) in April (12.6±1.37 ng/mL vs. 4.6±0.29 ng/mL). Sex-related changes in plasma ACTH concentrations were not observed (F=0.01, df=1, P=0.90), but a tendency in the influence of season was noted (F=3.28, df=2, P=0.05). ACTH levels (Fig. 2C) were higher ($P<0.05$) in females in November (15.1±1.14 pg/mL, n=5) than in April (13.0±0.42 pg/mL, n=5) and July (12.83±0.61 pg/mL, n=6).

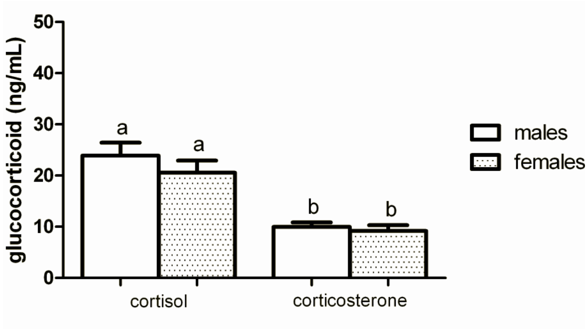

Fig. 1. A comparison of the mean plasma cortisol and corticosterone concentrations (ng/mL) in 18 male and 16 female beavers. Bars without common superscripts designate significant differences (P<0.05).

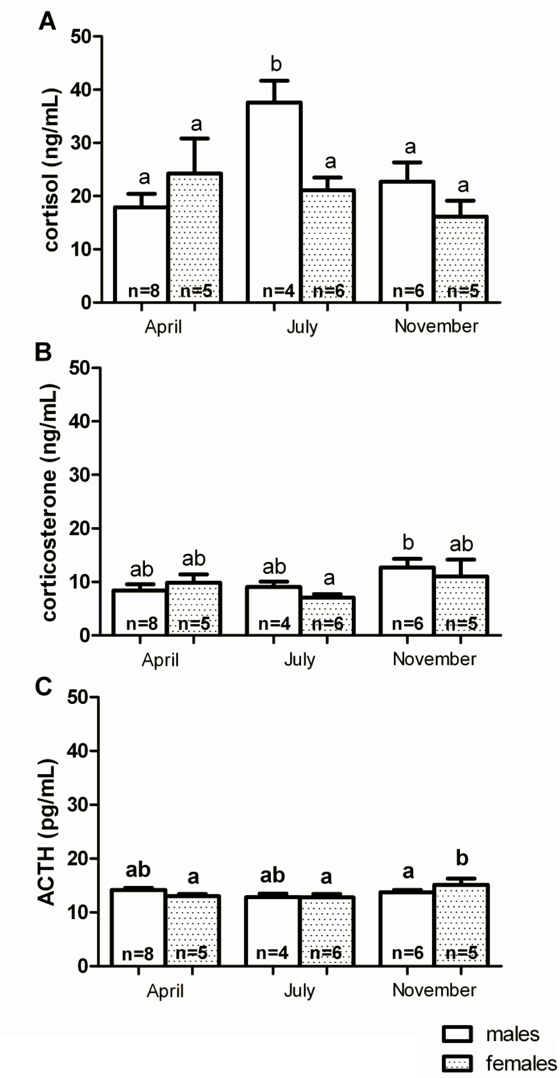

Fig. 2. Plasma cortisol (A), corticosterone (B) and ACTH (C) concentrations in a population of adult beavers during different seasons – April (8♂♂, 5♀♀), July (4♂♂, 6♀♀) and November (6♂♂, 5♀♀). Bars without common superscripts designate significant differences (P<0.05).

Discussion

Our experiment aimed to analyse adrenal function in free-living Eurasian beavers (*Castor fiber L.*). This is the first-ever study to demonstrate the type and concentrations of GCs and ACTH produced in beavers, and seasonal changes in their stress-induced plasma levels. Due to protection of the species, the number of individuals is relatively small. Beavers were caught from different habitats for a period of two years (2011/2012) by the same group of qualified staff.

Plasma GC and ACTH concentrations varied in response to stress associated with handling: capture, placing in cages and transportation to the laboratory. Unfortunately, it was impossible to obtain blood samples from free-living beavers under field conditions. Therefore, the true baseline levels of GCs were not available. Thus, our findings were discussed and compared only with the results of other relevant studies in which animals were also exposed to distressing events such as harvesting, transportation, immobilization or anesthesia. In our experiment, the total corticosteroid level was measured due to the relatively long blood collecting period. Determination of the total GC concentration is much better supported than estimation of free glucocorticoids and is less prone to error (SHERIFF *et al.* 2011; SCHOECH *et al.* 2013). Interestingly, BOONSTRA *et al.* (2001) showed an increase of total (unconjugated and conjugated with corticosteroid-binding globulin) and free (unconjugated) plasma cortisol levels (without changes in corticosteroid-binding globulin concentrations) as the effect of trapping and handling. However, activity, availability and fluctuating levels of total, free GCs and corticosteroid-binding globulin are still discussed (SCHOECH *et al.* 2013; BREUNER *et al.* 2013).

Our results indicate that the adrenal glands of beavers secrete both cortisol and corticosterone, but stress-induced plasma cortisol concentrations were about two-fold higher than corticosterone levels. We suggest that cortisol is the predominant corticoid in beavers exposed to stress. Simultaneous secretion of cortisol and corticosterone, with many-fold cortisol increase, has also been reported in other rodent species, including yellow-pine chipmunks (*Tamias amoneus*) and arctic ground squirrels (*Spermophilus parryii*) (BOONSTRA *et al.* 2001; KENAGY & PLACE 2000; PLACE & KENAGY 2000; ROMERO *et al.* 2008). Additionally, extremely high stress cortisol levels and traces of corticosterone were observed in degu (*Octodon degus*), (KENAGY *et al.* 1999). In turn, in golden hamster (*Mesocricetus auratus*), another dual secretor, corticosterone was the main stress corticoid (KOLLACK-WALKER *et al.* 1999). On the other hand, only corticosterone secretion has been observed in rats (*Rattus norvegicus, Rattus lutreolus velutinus*), house mice (*Mus musculus*), brown lemmings (*Lemmus trimucronatus*) and meadow voles (*Microtus pensilvanicus*) (AMIRAT *et al.* 1980; GALEA & MCEWEN 1999; LE GOASCOGNE *et al.* 1991; MONAMY 1995; ROMERO *et al.* 2008). Thus, considerable differences exist in rodent species connected with the secretion of different types of GCs in various proportions.

In the current experiment, plasma cortisol concentrations in beavers were affected by the season. In males, plasma cortisol levels varied throughout the year, to reach the highest value in July (post-breeding, offspring rearing), compared with April (gestation) and November (pre-breeding). Similar results were reported in male degus, living with females (late gestation and lactation) and caring for offspring (BAUER *et al.* 2014; KENAGY *et al.* 1999) as well as in a mixed population (males and females) of yellow-bellied marmots (*Marmota flaviventris*) but scientists do not know much about parental care in this species (ARMITAGE 1991). On the other hand, in males that do not contribute to the care of the juveniles, an increase of stress cortisol in post-breeding (yellow-pine chipmunks) and during breeding (golden-mantled ground squirrels) periods was also observed (BOSWELL *et al.* 1994; NUNES *et al.* 2006; PLACE & KENAGY 2000; ROMERO *et al.* 2008). Interestingly, no seasonal variation (from April to July) in free, corticoid-binding globulin and total cortisol levels were noted in male arctic ground squirrels, in which paternal investment is minimal and may be restricted to territory defense (BOONSTRA *et al.* 2001). Unlike the males, female beavers did not show seasonal changes in stress cortisol secretion. Similar results were obtained in arctic ground squirrels (BOONSTRA *et al.* 2001) and yellow-pine chipmunk (KENAGY & PLACE 2000) while an increase in cortisol concentrations during the breeding season was reported in female golden-mantled ground squirrels (BOSWELL *et al.* 1994). No seasonal and sex-dependent changes in stress-induced plasma corticosterone levels were observed in male and female beavers. However, corticosterone levels tended to be higher in males in November than in males in April. Lack of seasonal variation in corticosterone concentrations were found in female yellow-pine chipmunks (KENAGY & PLACE 2000). On the other hand, an increase in corticosterone secretion during the post-breeding period was reported in male yellow-pine chipmunk (KENAGY *et al.* 1999; PLACE & KENAGY 2000) as well as in male and female golden-mantled ground squirrels during the breeding season (BOSWELL *et al.* 1994). Summarizing these results, beavers belong to a group of animals that are characterized by dual secretion of cortisol and corticosterone, with cortisol being the main glucocorticoid. Seasonal changes in the secretion of stress-induced GCs in free-living rodents are also observed in beavers. Since GC levels are variable in different species and in various physiological states, it is difficult to draw a firm conclusion. We suggest that changes may also be dependent on social behavior (e.g. offspring rearing). The highest stress cortisol level in male beavers in the post-breeding period may be associated with the pre--sence of offspring and related territory defense. The lack of differences in plasma corticosterone levels may be connected with the fact that in species whose adrenals secrete both GCs, cortisol is the predominant hormone with higher activity (SHERIFF *et al.* 2011).

Our findings showed that stress-induced ACTH levels in free-living beavers remained constant regardless of the sex. It should be noted however that there was a tendency towards higher ACTH levels in females in fall (November, pre-breeding). Variation in ACTH level was not followed by changes in GCs. Very little is known about the circannual changes in ACTH levels in free-living rodents. For this reason, the discussion is limited. For example in free-living sand rats (*Psammomys obesus*) the secretion of ACTH was highest in summer (AMIRAT & BRUDIEUX 1993). Similar results were obtained in stallions (*Equus caballus*), pregnant and non-pregnant mares, in which the highest ACTH levels were observed in the summer and fall CORDERO *et al*. 2012; DONALDSON *et al*. 2005; FUNK *et al*. 2011). In contrast, no seasonal changes in ACTH concentrations were reported in stallions (SCHREIBER *et al*. 2012), as well as in a mixed population of male and female pigs (*Sus scrofa domestica*). Similarly, OTSUKA *et al.* (2012) did not show changes in ACTH levels under long- and short-day conditions in Fischer 344 rats. We suggest that changes in stress-induced ACTH and GC levels in beavers, similar to others species, might depend on variable environmental factors (season, temperature).

In conclusion, our study is the first to demonstrate that free-living beavers produce both cortisol and corticosterone, and cortisol seems to be the primary hormone associated with stress. Changes in the secretion of stress cortisol are seasonal and its highest levels were noted in males during the post-breeding period (offspring rearing). Corticosterone and ACTH levels in beavers remained generally constant, regardless of the season and sex. The unexpected lack of connection between stress-induced plasma ACTH and GC levels, observed in beavers, seasonal disconnection of the hypothalamic-pituitary-adrenal axis.

Acknowledgments

The authors would like to thank to Jan GOZDZIEWSKI from the Polish Hunting Association in Suwalki for capture and delivery of animals, Grzegorz BELZECKI, PhD. from The Kielanowski Institute of Animal Physiology and Nutrition of Polish Academy of Sciences in Jabłonna and Zygmunt GIZEJEWSKI, Prof. from Research Station of Ecological Agriculture and Preservation Animal Breeding of Polish Academy of Sciences in Popielno for cooperation and sharing of materials.

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