Leptin/leptin receptor system in the regulation of reproductive functions and stress response in the European beaver

Katarzyna Chojnowska, Joanna Czerwinska, Tadeusz Kaminski, Barbara Kaminska, Aleksandra Kurzynska, and Iwona Bogacka*

Department of Animal Anatomy and Physiology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Oczapowskiego Street 1A, Olsztyn 10-719, Poland

*Address correspondence to Iwona Bogacka. E-mail: iwonab@uwm.edu.pl.

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Abstract

The European beaver (Castor fiber L.) is the largest free-living rodent in Eurasia. The present work aimed to determine sex- and season-related changes in leptin receptor (Ob-R) expression in the hypothalamic–pituitary–gonadal/adrenal axes and uterus of beavers during breeding- (April), post-breeding- (July), and pre-breeding- (November) periods. The expression of Ob-R gene and protein was found in all analyzed tissues. The expression of Ob-R mRNA remained constant in the hypothalamus of both sexes during the analyzed stages. Sex- and season-related changes were found in the pituitary gland; the greatest level was observed in July in both sexes. The same expression pattern was noted in the testis, whereas in the ovary a lack of seasonal changes was found. In uterine tissues, the greatest expression occurred in November. The impact of season was also demonstrated in the adrenal cortex. In females, a higher Ob-R transcript level was noted in April, while in males, an increased mRNA abundance was noted in November than July. Our study suggests that in the beaver, leptin acting via the Ob-R can be an important endocrine factor engaged in the regulation of reproductive functions and stress response.

Key words: leptin receptor (Ob-R), beaver, seasonal breeding, hypothalamic–pituitary–gonadal axis (HPG), hypothalamic–pituitary–adrenal axis (HPA)

The European beaver (Castor fiber L.) is the largest free-living rodent in Eurasia. In the past, this species was formerly widely distributed across forested areas from western borders of Europe to eastern Siberia. Beavers are often referred to as an ecosystem engineers due to their ability to form or change existing habitats. They enlarge biodiversity and prepare the ecosystem for the emergence of various species of plants and animals. The animals exhibit a seasonal pattern of reproduction and they are referred as a long-day breeders with the peak reproductive activity occurring at the end of winter. Mating takes place in January and February (they mate under ice) and pregnancy lasts 105–107 days until May and June. During summer, beavers take care of pups and store food reserves. They accumulate subcutaneous fat deposits, but they do not hibernate (Zurowski 1992).

Although ecological aspects of beaver life have been broadly described, their physiology remains poorly understood. Our recent findings indicate season- and sex-related changes in beaver plasma sex steroids, glucocorticoids, and leptin concentration (Chojnowska et al. 2015; Czerwinska et al. 2015). The results also present changes in leptin mRNA abundance in the tissues of the hypothalamic–pituitary–gonadal/adrenal axes (HPG/HPA) and uterus, depending on season and sex of beavers (Chojnowska et al. 2017).

Studies performed on rodents and domestic animals revealed that leptin receptors are widely localized in several regions of the
brain that are involved in regulation of both energy balance and re-
production. Their presence has been also reported in various periph-
eral tissues, including reproductive tissues and the adrenal gland (for
review see Spicer 2001; Malendowicz et al. 2007). A number of
studies indicate leptin as a factor linking energy homeostasis, feeding
behavior, and reproductive functions (for review see Zieba et al.
2008).

We hypothesize that gene expression of leptin receptor varies in
the tissues of both regulatory axes (HPG and HPA) as well in the
uterus, depending on season and sex of beavers. Thus, the present
study aimed to investigate Ob-R mRNA abundance (determined by
quantitative real-time PCR) and protein localization (determined by
immunohistochemistry) in the structures of both axes and the uter-
us, depending on sex and reproductive stage.

Materials and Methods

Animals
The study was performed on 34 European beavers during 3 different
stages of their reproductive activity: April—“breeding period,” preg-
nancy in females (8 males, 5 pregnant females); July—“post-
breeding,” the end of lactation and raising of offspring (4 males, 6
females); and November—“pre-breeding,” sexual silence (6 males, 5
females).

Beavers were anesthetized with 2 anesthetic drug intramuscular-
ly with injections of xylazine (3 mg/kg of BW; Sedazin®, Biovet
Pulawy, Poland) and ketamine (15 mg/kg of BW; Bioktan,
Vetoquinol Biowet, Poland), and scarified. The pregnancy of females was confirmed by postmortem, by the presence of fetuses in the
uterus. Tissue samples [the medio-basal hypothalamus (MBH),
whole pituitary gland, testes, ovaries, the middle part of the uterine
horn divided into endometrium and myometrium, adrenal glands
from which adrenal cortex was separated, and subcutaneous white
adipose tissue (WAT)] were collected. The experimental material
was immediately frozen in liquid nitrogen and stored at −80°C until
further analysis.

Sequencing of Ob-R and quantitative real-time PCR
For the analysis of Ob-R gene expression, total RNA was extracted
from each tissue using an A&A Mini-column Kit (A&A Biotechnology,
USA) including the DNase treatment step. The concentration and qual-
ification of isolated RNA were determined spectrophotometrically
(Infinite M200 PRO, Tecan, Switzerland) and integrity was verified on
1.5% agarose gel. The obtained RNA was reverse-transcribed into
dNA determined by electrophoresis on 1.5% agarose gel. After extraction
from the gel (GenEluteTM Gen Extraction Kit, Sigma, USA), DNA
was sequenced (Genomed S.A., Warsaw, Poland), DNA
sequence from the gel (GenEluteTM Gen Extraction Kit, Sigma, USA), DNA
was sequenced (Genomed S.A., Warsaw, Poland) in both directions.

The partial sequence of leptin receptor cDNA was determined
based on rat (NM_012596.1), mouse (NM_146146.2), pig
(NM_001024587.1), and human (NM_002303.5) leptin receptor
sequences. For analysis, the most conservative sequence regions
were chosen in the extracellular domain of the receptor by using the
Basic Local Alignment Search Tool (BLAST). The final primer sequence
set is presented in Table 1A. The PCR amplification was performed
using JumpStart™ (Sigma, USA). PCR-amplified DNA was
determined by electrophoresis on 1.5% agarose gel. After extraction from the
gel (GenEluteTM Gen Extraction Kit, Sigma, USA), DNA
was sequenced (Genomed S.A., Warsaw, Poland) in both directions.

Quantitative real-time PCR analysis was carried out using a PCR
System 7300 and Power SYBR Green Master Mix (Applied
Biosystems, USA), and specific primer pairs (Table 1B) used to ampl-
ify parts of Ob-R were designed after sequencing results. The β-
actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) genes (Chojnowska et al. 2017) were used as internal con-
trols. In no-template controls, the cDNA was replaced by RNase-
free water. The specificity of amplification was tested at the end of
the reaction by analyzing the melting curve. The relative expression
of leptin receptor was calculated with the use of the comparative
cycle threshold method (ΔΔCt) as described previously
(Chojnowska et al. 2017).

Immunohistochemistry
Immunohistochemical analysis was performed as described previ-
ously (Chojnowska et al. 2017). The collected frozen tissues were
cut using a cryostat CM3050 (Leica, USA) and mounted onto
poly-t-lysine-coated glass microscope slides (Menzel-Glaser,
Braunschweig, Germany). The sections were incubated with primary
rabbit polyclonal antibodies against leptin receptor (1:50, Abcam®
UK) and with secondary anti-mouse/rabbit antibodies (ImmPRESS
Universal Reagent Anti-Mouse/Rabbit Ig, Vector Laboratories,
USA). To visualize the immunoreactivity, the sections were
immersed in 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako,
USA), and then counterstained with hematoxylin (Aqua-Med,
Poland). The labeled tissues were photographed using a C-5060
Camera (Olympus, Japan) mounted on a light microscope (CH30/
CH40, Olympus, Japan). For negative controls, the tissue slices
were incubated in 0.01 M PBS instead of primary and/or secondary
antibodies.

Statistical analysis
Statistical analysis for leptin receptor mRNA abundance in the tis-
ues that were assessed within each experimental group (males or
females in different reproductive stages) was performed using
Statistica software (Statsoft Inc., Tulsa, USA). Compatibility with
the normal distribution of each variable was tested with the Shapiro–
Wilk’s test. The data were analyzed by one-way ANOVA followed
by the Tukey’s post hoc test and are presented as means ± SEM. To
evaluate the impact of sex and/or season on leptin transcript expres-
sion, a two-way ANOVA analysis was performed. The relation be-
 tween levels of Ob-R mRNA and Ob mRNA as well as Ob-mRNA
and plasma leptin was described by Pearson’s correlations coeffi-
cient. Values P < 0.05 were considered as statistically significant.

Results
The obtained PCR product contained 318 bp. The sequence exhib-
ted homology with the European rabbit (Oryctolagus cuniculus,
XM_008265114.1; 92% identity), rat/mouse (NM_012596.1/
NM_146146.2; 85%/86% identity), pig (NM_001024587.1; 88%
identity), and with human (NM_001198689.1; 91% identity) leptin
receptor sequences (Figure 1).

The presence of Ob-R mRNA and protein was noted in all tested
beaver tissues (the MBH, pituitary gland, ovary, testis, uterus, ad-
renal gland, and WAT) during the analyzed reproductive periods.
Two-way ANOVA analysis revealed no impact of sex, season, or
the interaction between sex and season in the MBH (Figure 2A,B).
The presence of Ob-R protein was confirmed in the MBH during the
tested stages (the representative data are presented in Figure 3A).

In the pituitary gland, the impact of sex (F = 12.08; P < 0.05), season (F = 28.11; P < 0.05), and their interaction
(F = 6.51; P < 0.05) on Ob-R mRNA expression was assessed. In
both males (Figure 2C) and females (Figure 2D), the highest
Table 1. The sequences of primers used for identification of leptin receptor (Ob-R) cDNA in PCR (A) or leptin receptor and reference genes: β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in quantitative real-time PCR (B)

| Gene name | Primer sequences (5′–3′) | \( T_m \) (°C) | Product length (bp) |
|-----------|--------------------------|----------------|---------------------|
| (A) PCR   |                          |                |                     |
| Ob-R      | F: CAAGCATACAGCATGATGCTAG | 60             | 318                 |
|           | R: CTGCTAGAGAAGCATTTGCTGACTG |               |                     |
| (B) Real-time PCR |                        |                |                     |
| Gene name | Primer sequences (5′–3′) | \( T_m \) (°C) | Product length (bp) |
| ACTB      | F: ATCGCCGACAGATGCA      | 60             | 102                 |
|           | R: CGTACTCTGCTGCTGCTGATCC |               |                     |
| GAPDH     | F: CCTTCATTGACCTCCACTAC  | 59             | 123                 |
|           | R: CCACAACATACGTACACCCA  |               |                     |
| Ob-R      | F: CAGATTGTCAGCAAATACAATC | 60             | 112                 |
|           | R: CTCAGATATGCTTGTGAGCATGTC |            |                     |

Figure 1. The sequence of leptin receptor in beavers and its homology with the European rabbit (XM_008265114.1), rat (NM_012596.1), mouse (NM_146146.2), pig (NM_001024587.1), and with human (NM_001198689.1) Ob-R sequences.
(P < 0.05) abundance of Ob-R mRNA was noted in July (post-breeding) compared with April (breeding) or November (pre-breeding). The Ob-R protein presence was observed in the pituitary gland collected from all analyzed reproductive stages (the representative data are presented in Figure 3B).

In the testis (Figure 2E), a greater relative abundance of Ob-R was demonstrated in July in comparison with November. In turn, in the ovaries (Figure 2F), no seasonal changes in the expression of Ob-R mRNA were noted. The Ob-R protein was localized in the testis in smooth muscle layer, Sertoli-, Leydig-, and myoid cells. In ovaries, Ob-R protein was observed in cells forming stroma and primordial and primary follicles (the representative data are presented in Figure 3C,D, respectively).

In the uterus, a similar pattern of Ob-R mRNA content was observed in both myometrium (Figure 2G) and endometrium (Figure 2H). The greatest content of Ob-R transcript was found in November (pre-breeding). The Ob-R protein was localized in both the endometrium (uterine glands and luminal epithelium layer) and myometrium (circular and longitudinal muscle layers); the representative data are presented in Figure 3E.

In the adrenal cortex, the impact of season (F_{2,23} = 18.11; P < 0.05) and interaction between sex and season (F_{2,23} = 19.08; P < 0.05) were found. In females (Figure 2J), a higher Ob-R transcript level was noted in April (pregnancy) when compared with the remaining periods. In males (Figure 2I), an increased mRNA abundance was noted in November (pre-breeding) in comparison with July (post-breeding). The Ob-R protein was detected in both the cortex (glomerulosa, fasciculata, and reticularis layers) and medulla (the representative picture is presented in Figure 3F).

A lack of changes in Ob-R mRNA abundance in subcutaneous WAT was observed, regardless of either sex or season impact (Figure 2K,L).

Generally, we did not found many important correlations between the analyzed parameters. The most significant correlation was noted between levels of mRNA for OB-R and OB in the male MBH in April (r = 0.97; P < 0.01).

**Discussion**

The beaver has been frequently studied as model for behavioral ecology and ecosystem engineer for many years. Despite considerable interest in a strong influence on the environment and reintroduction programs, little is known about physiology of the beaver. Beaver tissues/organisms remain insufficiently studied with respect to their functions and in comparison with other wild species with seasonal breeding. The present study demonstrates, for the first time, the expression of gene and protein of leptin receptor in the structures of the HPA and HPG axes as well as in the uterus of the European beaver. This is also the first report indicating sex- and season-related changes in Ob-R mRNA expression in the above structures. The expression of Ob-R mRNA remained constant in the hypothalamus of both sexes during the analyzed stages. Sex- and season-related changes were found in the pituitary gland; the greatest level was observed in July in both sexes. The same expression pattern was noted in the testis, whereas in the ovary a lack of seasonal changes was found. In uterine tissues, the greatest expression occurred in November. The impact of season was also demonstrated in the adrenal cortex. In females, a higher Ob-R transcript level was noted in April, while in males, an increased mRNA abundance was noted in November than July.
The study regarding distribution and physiological role of leptin/leptin receptor system in various animal species was widely discussed, although in protected free-living animals, including the beaver, it is often problematic and limited due to difficulties in obtaining adequate samples representing all reproductive stages. While the presence of Ob-R mRNA/protein in various levels of the HPG/HPA axes has been described in laboratory rodents or domestic animals, there have been few studies conducted on wild rodents such as Syrian Mesocricetus auratus and Siberian hamsters Phodopus sungorus (Mercer et al. 2000), Brandt vole Lasiodipodomys brandti (Zhang et al. 2011), and Daurian ground squirrel Spermophilus dauricus (Xing et al. 2015).

There is evidence that the photoperiod in wild living animals affects the expression of Ob-R. It has also been shown that a long day augments the expression of long form of Ob-R mRNA in the hypothalamus of both sexes of juvenile Siberian hamsters whereas a short day exerts an opposite effect (Adam et al. 2000; Mercer et al. 2000). Unfortunately, such a tendency was not confirmed in our study, since Ob-R expression did not alter in the hypothalamus of females during the analyzed reproductive stages. In male hypothalamus, Ob-R mRNA abundance in November (pre-breeding; short day) remained constant when compared with July (post-breeding; long day). In turn, a higher level was noted in April (breeding). In the male MBH in April the correlation was noted between levels of mRNA for OB-R and OB.

Interestingly, the above findings can be associated with the results regarding to leptin serum concentration obtained from the same group of beavers (Chojnowska et al. 2017). Although the plasma leptin concentration remained stable during the analyzed stages in females, changes were noted in males. We may assume
that, in males, a low peripheral leptin concentration in April (breeding) up-regulates (whereas a high plasma leptin content in July—post-breeding down-regulates) the expression of Ob-R in the MBH. Additionally, our previous findings reported seasonal differences in leptin gene expression that may suggest possible local regulatory impact of leptin on the receptor mRNA abundance in the MBH of beavers (Chojnowska et al. 2017). The changes in Ob-R expression may imply a different leptin availability in the brain.

Sex- and season-related changes in Ob-R mRNA abundance were found in the pituitary gland of beavers. Interestingly, the pattern of the expression was similar in both females and males—the greatest mRNA abundance was found during the post-breeding season (July) when compared with breeding (April) or sexual silence (November) periods. It is also worth mentioning that the present results and our previous findings show similar expression pattern of Ob-R mRNA and leptin mRNA (Chojnowska et al. 2017) in the pituitary gland as well as FSH plasma concentration (Chojnowska et al. 2015) in females in July and April. However, such relation cannot be observed in males. Despite similarity in Ob-R mRNA expression in the pituitary gland with females, we did not find any relation with plasma gonadotropins concentration or leptin synthesis in this tissue (Chojnowska et al. 2017). In addition, we did not observe any consistency with plasma testosterone concentration during the analyzed reproductive stages (Chojnowska et al. 2015) although a very similar profile of Ob-R mRNA expression was also noted in the testis when compared with the pituitary.

The Ob-R mRNA abundance in the ovary was relatively constant through the tested reproductive periods, whereas both the endometrium and myometrium showed season-dependent changes—the greatest level was in November (pre-breeding). Our results concerning the ovary are, to some degree, surprising because experiments conducted on the ovary of the human, pig, or dog revealed that Ob-R mRNA expression varied depending on the stage of the menstrual/estrous cycle or pregnancy (Cervero et al. 2004; Smolinska et al. 2013; Balogh et al. 2015). The Ob-R protein has been identified in beaver ovarian structures, i.e., corpora albicantia and primordial and primary follicles. Similarly, the Ob-R protein was localized in ovaries of the rat and mouse (Ruiz-Cortez et al. 2000; Ryan et al. 2002). In turn, the finding regarding the presence of Ob-R protein in beaver uterine tissues including structures, such as uterine glands and luminal epithelium layers as well as circular and longitudinal muscle layers, is supported by immunohistochemical Ob-R staining in uteri of the mouse (Kawamura et al. 2002), rat (Plastow and Waddell 2002), dog (Balogh et al. 2015), as well as in Japanese black bear (Nakamura et al. 2009).

Season-associated changes in Ob-R expression were observed in the beaver’s adrenal cortex. Elevated abundance of Ob-R and leptin mRNAs (Chojnowska et al. 2017) was noted in females in April (pregnancy) than in July (post-breeding). In turn, in males, higher Ob-R and leptin mRNA levels were noted in November (pre-breeding) than in July (post-breeding). The presence of Ob-R gene and protein in all branches of the HPA axis suggests that leptin can modulate the stress response in this species. It is known that leptin suppresses glucocorticoid secretion by the adrenal cortex through the direct/indirect effect on hypothalamic CRF and pituitary ACTH release (for review see Rousos et al. 2012). There are also reports describing the direct inhibitory effect of leptin on cortisol secretion from bovine (Bornstein et al. 1997), rat, and human (Pralong et al. 1998) adrenocortical cells although leptin itself is not expressed in the human adrenals (Glasow et al. 1998).

The Ob-R protein, detected by immunocytochemistry, was strongly expressed in human cortical cells, whereas the adrenal medulla showed only a weak expression in cortical cell islets (Glasow et al. 1998). It should be pointed out that leptin itself is not expressed in the human adrenals (Glasow et al. 1998). Our findings indicate the presence of Ob-R (this study) and leptin (Chojnowska et al. 2017) mRNAs and proteins in the beaver adrenals of both sexes. Interestingly, elevated abundance of Ob-R and leptin mRNAs was observed in females in April (pregnancy) than in July (post-breeding). In turn, in males, higher Ob-R and leptin mRNA levels were noted in November (pre-breeding) than in July (post-breeding). It can be assumed that the lower mRNA abundance of leptin and Ob-R in the adrenal gland of males in July could be related to a higher plasma cortisol concentration during this stage (Czerwinska et al. 2015). This observation could support a negative relationship between leptin and cortisol plasma concentration, as discussed above. It is worth mentioning that plasma cortisol concentration in females remained constant during the analyzed reproductive stages (Czerwinska et al. 2015). The above findings suggest that leptin can participate in the regulation of the HPA axis on a sex-specific basis and the exact role of leptin in functioning of the HPA axis in both sexes needs to be explored.

In conclusion, this study indicates the presence of Ob-R gene and protein expression as well as sex- and/or season-related changes in Ob-R mRNA abundance in all structures of the HPG/HPA axes and uterus of the European beaver. The observed circannual changes in leptin receptor mRNA abundance can be associated with the differential activity of the HPG/HPA axes during different reproductive stages. A lack of common pattern in leptin receptor gene expression in male and female beavers suggests a gender-specific biological role of leptin which can be an important link connecting metabolism, reproductive processes, and stress response.

Ethics Statement
All experimental procedures were conducted in accordance with ethical standards of the institutional Animal Ethical Committees [ministerial approval: RDOŚ-28-OOP-6631-0007-638/09/10/pj and local approvals: SGGW/11/2010 and UWM/87/2012/DTN].

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