Reproductive cycle of the domestic horse (Equus caballus) is strongly characterized by seasonal breeding. Mares show oestrus symptoms during the prolonged daylight, while the activity of the stallion's reproductive system is observed throughout the year. However, there are noticeable differences in the quality of stallion's semen obtained from the period defined as the breeding season (April-September) and out of the breeding season (autumn and winter months). Out of the breeding season, sperm concentration, motility and viability parameters decrease, as well as the volume of ejaculate decreases. Repeatedly, during the sport horses breeding, sudden accidents eliminating valuable stallions from further breeding occur. Therefore, the interest in using the epididymal spermatozoa, which is a reservoir of genetic material is constantly increasing. The analysis of motility and morphology of epididymal and ejaculated spermatozoa did not show significant differences, which makes it possible to use the epididymal sperm in the event of a sudden death of a valuable stallion or forced castration.

Stallion's epididymis is anatomically divided into three segments. Spermatozoa located in the caput of epididymis, wich provides mainly secretory functions, are immature and do not have an ability to fertilize the oocyte. Following segments of epididymis - corpus and cauda act as the sperm reservoir. The specific mileu of corpus and cauda epididymis maintain sperm viability, protect it from the oxidative stress, inflammation and temperature. All the biochemical and morphological changes that occur during the passage of spermatozoa through the epididymal duct are defined as the epididymal maturation. They relate to the reorganization of lipids and membrane proteins composition that arise from the change in the distribution or removal of some components during the action of proteolytic enzymes. Such enzymes can easily detach oligosaccharide residues and attach de novo synthesized proteins to the sperm during its passage in the epididymis.

Protein phosphorylation is one of the most important reactions during the sperm maturation. This process regulates the activity and function of many polypeptides i.e. it regulates cell activity, controls cell metabolism, apoptosis and protein ubiquitination.

There is no data available in the literature concerning the intensity of phosphorylation of the stallion epididymal semen proteins. Moreover, there is no

information about identified phosphopeptides from stallion's epididymal fluid and sperm extracts.

The aim of this study was to characterize phosphoproteome of stallion epididymal fluid and sperm extracts in two distinguished periods of breeding season with use of chosen proteomic techniques. The research material were epididymides obtained from 12 mature stallions during routine castration procedures. The phosphoproteins were isolated by immobilized metal-ion affinity chromatography, next they were separated by 1D-PAGE electrophoresis and finally identified by NanoLC-MS/MS mass spectrometry. Additionally, degree of phosphorylated state was analyzed by immuno- and western blot techniques.

Analysis of the 1D PAGE electropherograms of epididymal fluid phosphoproteins, obtained during the breeding season showed the presence of ten, common fractions for every of the epididymis segments (with molecular weights 76, 66, 58, 55, 49, 47, 44, 34, 31 i 22 kDa). Two fractions were present mainly in the caput and corpus (136, 92 kDa), whereas one was found mainly in the cauda of the epididymis (116 kDa). Phosphopeptide profiles, isolated out of the breeding season revealed the presence of three phosphoproteins in the caput and corpus of the epididymis (136, 128 i 92 kDa). In addition, fifteen proteins were present in each epididymal segment (76, 66, 58, 55, 49, 44, 34, 31, 29, 25, 24, 22, 19, 15 i 14 kDa). Fourteen phosphoproteins undergo phosphorylation both during and out of the breeding season (136, 128, 116, 92, 76, 66, 58, 55, 53, 49, 47, 44, 38, 34, 31, 29, 25 i 24 kDa). The highest number of phosphoproteins was found in sperm extracts from the caput and corpus regardless the reproductive season. Decrease in the number of phosphorylated proteins was observed in each segment out of the breeding season. Twenty-eight phosphoprotein fractions were observed (280, 160, 150, 110, 100, 83, 75, 73, 70, 61, 57, 50, 48, 44, 41, 35, 32, 31, 29, 26, 25, 24, 23, 22, 17, 16, 15 i 12 kDa), which presence varied between individuals and epididymal segments.

The proteins identified in the hereby study, mainly antioxidative enzymes (i.e. ceruloplasmin, peroxiredoxin -1, -2, -5, serotransferrin, glutathione-s-transferase mu 5) and heat shock proteins (HSP 70, HSP β1), play a crucial role in the regulation of stallion's reproduction processes. Their protective functions against the harmful

products of redox reactions, regulation of spermatogenesis and sperm maturation in epididymis can be useful to explain the molecular mechanisms that regulate equine reproduction.