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### **CORTISOL AS A MARKER OF PHYSIOLOGICAL STRESS IN *Bos taurus* SIRE**

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#### **Abstract**

The endocrine mechanism in animal stress has not yet been fully elucidated. Social stress due to hierarchy in the herd increases the production of cortisol, catecholamines, can affect sexual behavior and fertility, and reduce productivity. Despite the enormous importance of the problem for breeding enterprises involved in the production and cryopreservation of sperm of farm animals for commercial and scientific purposes, there are no special studies to investigate the connection of the qualitative and quantitative parameters of the ejaculate and its fertilizing ability with the post-ejaculatory cortisol level. In the presented work, we revealed for the first time that the effectiveness of artificial insemination is directly proportional to the concentration of blood cortisol in stud bulls after ejaculation. In young stud bulls, stress associated with blood sampling dominated; in adult bulls, sexual arousal suppressed stress caused by this veterinary manipulation. For the first time, we find out the dependence of the fertilizing capability of the bull sperm on the post-ejaculation blood cortisol level. We assessed the pattern of post-ejaculation cortisol concentration in 11 Holstein bulls of foreign selection aged 29-71 months (holding JSC GCV, a subsidiary breeding enterprise of JSC Uralplemstentr, Sverdlovsk region, 2018-2021) which are exploited according to approved technology and determined the fertilizing rate of the cryopreserved resulting sperm as influenced by the blood cortisol level. In both young and adult animals, there was a significant positive dynamics of cortisol in response to blood sampling. The average blood cortisol concentration in young sires ( $n = 6$ ) under stress from this regular manipulation increased evenly and reached 70.8; 91.1 and 144.7 nmol/l on the days of three consecutive doublet collections of semen with 4-5-day intervals. In bulls ( $n = 5$ ) older than 4 years, the peak concentration of cortisol (172.5 nmol/l) occurred in the second semen collection, and by the third collection, there was a slight decrease in the cortisol level, by 5 %. As post-ejaculation blood cortisol increases, the number of spermatozoa per ejaculate increases. In young bulls, after three consecutive double collections of semen at 4-5 day intervals, 4.0, 5.5 and 6.2 billion spermatozoa per ejaculation were obtained, in sires over 4-year old, 6.6, 7.3 and 7.5 billion sperm. The sperm from sires with the highest post-ejaculation levels of cortisol released into the bloodstream (162-216 nmol/l) exhibited the best fertilizing rate, 59-60 % ( $p < 0.001$ ).

Keywords: sires, cortisol, ejaculation, sperm production, stress, artificial insemination

Modern intensive technologies, specialized farming and high livestock density do not mostly correspond to the evolutionary physiological features of domesticated animals. Therefore, ensuring animal welfare, in particular avoiding chronic stress, is becoming increasingly important. Stress is a result from an influence that poses a load on a biological system [1]. Physical (heat, noise, transportation, food restriction) and psychological (weaning, isolation, moving from group to group) reasons can cause stress [2, 3]. The effects of endogenous and

exogenous stress factors differ in both duration and characteristic features [4, 5].

R. Collier et al. [6] believe that stress causes behavioral, metabolic, and physiological changes in animals. Under stress, the body uses internal reserves to survive an unfavorable period during which physiological modulations occur in functions of the nervous system and various endocrine reactions are activated [7, 8].

The endocrine mechanism of stress and its influence on the interaction of the body with the environment has not yet been fully elucidated [9]. Exogenous factors (climate, feeding conditions, housing and industrial use of animals) are considered the main sources of stress that can cause various epigenetic modifications of traits [10]. Prolonged or intense exposure to physical, psychological or other stress factors causes an excessive response in which the main adaptive effects become damaging one. In combination with genetic factor, this leads to the acceleration and qualitative distortion of the natural mechanisms of biological aging, the formation of somatic pathology and even to premature death [11, 12].

Social stress due to herd hierarchy has been shown to activate the adrenal axis, increase the production of cortisol, catecholamines, and in the long term can affect fertility, suppress immunity, cause neurological dysfunction [13], and reduce productivity [14]. Under stress, sexual behavior is inhibited and even completely suppressed, which is due to a decrease in the secretion of gonadotropins by the pituitary gland and depends on hypothalamic releasing factors. Prolonged stress leads to serious disorders of sexual function [15-19] up to infertility [17, 20], causes physiological changes and reduces the body's resistance [18, 19, 21], activates oxidation mechanisms resulting in germ cell damage by aggressive free radicals [22].

Stress provokes the production of glucocorticoids which affect metabolism and reduce the sensitivity of tissues to sex hormones. In males, chronic stress leads to gonadal exhaustion, decreased testosterone production, and the development of secondary infertility [23, 24]. A decrease in the concentration of follicle-stimulating hormone (FSH) occurred under stress leads to impaired spermatogenesis, slower differentiation and proliferation of Sertoli cells and, as a result, to complications in the later stages of spermatogenesis. With a decrease in the level of luteinizing hormone (LH), the testosterone synthesis in Leydig cells decreases resulting in hypogonadotropic syndrome (secondary hypogonadism) which provokes depletion of the reserve capacity of the gonads [23, 24].

Cortisol is a biomarker of stress. According to a number publication, both in mammals in general [23, 24] and in breeding bulls [25] particularly, hormones of the hypothalamic-pituitary-gonadal and thyroid endocrine regulation can serve as the main tool of adaptation. The primary neuroendocrine system that controls stress responses and regulates many processes in the body, including digestion, sexual function, energy storage and expenditure, is the hypothalamic-pituitary-adrenal axis which provides coordinated interaction between glands, hormones and parts of the midbrain that mediate the overall adaptation syndrome [26, 27].

When adaptive mechanisms can no longer cope with the stressor, the hypothalamic-pituitary-adrenal axis prompts the hypothalamus to secrete corticotropin-releasing hormone and vasopressin [28]. They, in turn, induce the pituitary gland to release adrenocorticotrophic hormone (ACTH). Under its influence, glucocorticoids, mainly cortisol, are synthesized from cholesterol in the zona fasciculata of the adrenal cortex and then secreted into the bloodstream [29-31]. Factors that stimulate the release of cortisol are stress, hypoglycemia and exercise. All types of physical and psycho-emotional stress, within a few minutes of onset, can lead to an extreme secretion of ACTH and cortisol to neutralize the impact of the negative feedback system and circadian rhythm. Thus, physiological hypersecretion of corticotropin-releasing hormone and increased function of the entire hormonal axis of the hypothalamus-pituitary-adrenal cortex occurs in stress or with

increased activity [32].

Cortisol, in turn, binds to plasma globulins and especially albumin and corticosteroid binding globulin (CBG) and is transported by the circulatory system [33]). The effect of cortisol depends on how long and in what quantity cortisol is secreted, how much binding globulin is present in the peripheral blood, how many receptors are in target tissues, and the extent to which glucocorticoid metabolites have been broken down [34]. At high cortisol concentrations, further cortisol release from the adrenal cortex is inhibited by a negative feedback mechanism involving the hypothalamus and anterior pituitary gland, thereby abolishing the stress response [35].

Cortisol is sufficiently lipophilic to cross the plasma membrane of target cells, it binds to intracellular glucocorticoid receptors, and the resulting complexes translocate to the nucleus where they can either increase or decrease the expression of various genes.

Thus, cortisol can influence many important biochemical functions, including ACTH release, synthesis of gluconeogenesis-related enzymes, muscle protein breakdown, and lipolysis [29, 36].

Long-term stress can lead to high levels of circulating cortisol. In this case, a complex hormonal cascade occurs and the adrenal glands secrete cortisol which stimulates the supply of glucose as a source of rapid energy for large muscles in the implementation of the stress response. The adrenal glands increase the secretion of cortisol in the first stage of stress development (anxiety stage) during the first 6-48 h under the influence of adrenaline and norepinephrine. At the second stage (resistance), high cortisol levels are accompanied by functional changes that ensure adaptation to the stressor. Depletion of the adrenal cortex and, as a consequence, a decrease in cortisol levels occurs in the third stage, while opioid peptides put the body into maximum energy-saving mode [37].

Cortisol interferes with the action of insulin, essentially making cells resistant to it. The body remains insulin resistant when cortisol levels are chronically elevated. Over time, the pancreas has difficulty keeping up with the high demand for insulin, leaving blood glucose levels high and cells unable to get the sugar they need [38].

Metabolic changes that occur under the influence of cortisol determine successful adaptation to stress. When nervous tissue is stressed, a large amount of energy is required, which is provided by the synthesis of glucose from non-carbohydrate substrates [39]. Under the influence of cortisol, some skeletal muscle proteins break down to components from which glucose is synthesized. This is the catabolic effect of the hormone, which, during the period of adaptation to a stressor, provides more effective distribution of the body's energy resources [40]. However, such long-term metabolic restructuring negatively affects the body, therefore there are physiological mechanisms for inhibiting the synthesis of cortisol. They include a decrease in the production of corticoliberin under the influence of cortisol itself (negative feedback) and the simultaneous production of another steroid hormone by the adrenal glands, the dehydroepiandrosterone (DHEA), which has an effect opposite to cortisol. A constant blood level of DHEA prevents the psychological maladjustment and stress-induced diseases [41].

Cortisol, as a stress hormone, is characterized by a circadian rhythm with increased concentrations in the morning [25, 42, 43]. A complex relationship has been proven between disruption of circadian rhythms and metabolism. Alterations in the sleep-wake cycle can lead to metabolic syndrome [44].

Plasma cortisol exists in three forms. The majority (80-90%) is bound to the corticosteroid-binding protein transcortin (alpha-2-globulin), some is bound to albumin, and less than 10% of the hormone freely circulates. The biological activity of cortisol is due to its free form which can directly affect target cells [36].

Cortisol and other glucocorticoids in cattle regulate the balance between anabolism and catabolism, and under heat stress conditions they reduce the expression of lipoprotein lipase responsible for lipolysis, reduce the activity of carbohydrate metabolism, the number of peripheral blood leukocytes, and change the expression of genes associated with glycolysis and insulin-induced absorption of glucose. High levels of corticotropin-releasing hormone in tissues outside the brain can have potent inflammatory effects [1, 29].

Cortisol concentration increases during bull castration without local anesthesia [45], in mastitis [46], in inflammatory diseases of the hooves [47], and in stress caused by forced lying [48]. In cattle, prolonged increases in cortisol concentrations lead to elevated blood sugar levels and diabetes mellitus which is more common in young animals [49]. Higher serum cortisol concentrations have been found in cows with a negative energy balance compared to cows fed an ad libitum diet [50]. In highly productive dairy cows, the effect of the administration of adrenocorticotrophic hormone on cortisol synthesis is less pronounced than in low-producing dairy cows. One cause of lactation ketosis may be a problem with cortisol synthesis when energy demands increase during peak lactation [51]. Low cortisol levels have been detected in cows suffering from spontaneous ketosis [52]. However, hypoglycemia was not recorded in these animals [53].

The effects of cortisol on the adhesion and migration of leukocytes have been noted, and in stud boars (regardless of breed), with a decrease in cortisol concentration, the number of segmented neutrophils decreased [54]. An increase in the concentration of blood cortisol is associated with hypertriglycerinemia, increased protein-lipid and carbohydrate metabolism [19].

Regarding the effect of ACTH on the gonads, on the one hand, the hormone is nonspecific and promotes the synthesis of testosterone in Leydig cells, and on the other hand, it inhibits its release. This explains the phenomenon of suppression of androgen secretion in males under neurogenic stress [55].

Glucocorticoids inhibit reproductive function in most domestic animal species [56]. High stocking density (1.2 m<sup>2</sup> per animal) leads to a sharp increase in the concentration of blood cortisol in bulls and stunted growth compared to individuals kept in more spacious stalls (4.2 m<sup>2</sup> per animal).

During sexual activity in men, a positive relationship has been established between the levels of cortisol and testosterone with a simultaneous increase in concentrations [57, 58]. In adult male rhesus macaques, mean plasma concentrations of testosterone and luteinizing hormone did not increase significantly after intercourse or in control without ejaculation, whereas cortisol increased in both cases [59].

Seasonality of cortisol release after electroejaculation was observed in Syrian Awassi rams. Cortisol reached its maximum concentration 20 min after electroejaculation, with no significant difference between the two seasons [60]. Cortisol levels increased during arousal and ejaculation, and after electroejaculation in anesthetized animals during routine semen collection from beef bulls [56]. In horses and donkeys, cortisol concentrations increased abruptly 30 min after ejaculation [61, 62].

Thus, short-term cortisol surges are necessary to recover from the effects of stress and increase sugar levels as an energy substrate. Excessive sexual activity depletes the pool of hormones and nutrients, simultaneously increasing metabolic byproducts and stress hormones.

Despite the great interest in the topic, there are no special studies on the effect of post-ejaculatory cortisol on the quality and quantity of the ejaculate and the sperm fertilizing ability. The problem is of great importance for breeding enterprises which produce cryopreserved semen for commercial and scientific use, but remains poorly understood.

In this work, we discovered for the first time that the effectiveness of artificial insemination is directly proportional to the concentration of blood cortisol in stud bulls after ejaculation. In young breeding bulls the stress from blood sampling dominates, while in adults, sexual arousal suppresses the stress from this daily manipulation.

The purpose of the study is to determine the dynamics of blood cortisol concentration in Holstein bulls of different ages after ejaculation at the frequency of semen collection according to approved technology and to evaluate the effect of cortisol on the fertilizing ability of the resulting sperm after cryopreservation.

*Materials and methods.* Blood cortisol concentration was daily measured 11 Holstein bulls of foreign selection aged 29-71 months (holding company GCV JSC, the subsidiary breeding enterprise Uralplemstentr JSC, Sverdlovsk Province, 2018-2021). The animals had normal condition and a calm temperament.

Feeding, maintenance and exploiting of breeding bulls complied with the National technology for freezing and using sperm of breeding bulls [63]. Young animals aged 29-36 months were assigned to group I ( $n = 6$ ), bulls over 4 years old to group II ( $n = 5$ ). The sires were kept in the same conditions with a similar sexual load. The experimental duration was 14 days.

Venous blood to monitor cortisol levels was taken daily (except Saturdays and Sundays) for 2 weeks in the morning between 11 pm and 12 pm. From each bull, 7-8 ejaculates over the experiment were collected in a doublet twice a week according to the technological schedule, no earlier than 30 min before blood collection.

In 2018, blood samples were collected in disposable VacPlus vacuum tubes with EDTA tripotassium salt (Shandong Chengwu Medical Products Factory, China). The serum was separated by centrifugation at 3000 rpm for 5 min, the supernatant was poured into 20 ml cryovials and stored in a freezer at  $-18$  to  $-20$  °C until analysis for endogenous hormones performed in 2018. Cortisol concentration was measured by enzyme-linked immunosorbent assay (ELISA) in two analytical replicates with reagent kits as per the manufacturer's protocol (JSC Immunotech, Moscow) and a UniPlan spectrophotometer (AFG-01) (JSC Pikon, Russia).

Ejaculates obtained in 2018 were assessed for compliance with established requirements for undiluted bull semen (GOST 23745-2014). Ejaculates that met the standards were diluted with OPTIXcell cryoprotector (IMV Technology, France), packed in 0.25 ml paillettes and cryopreserved on equipment from IMV Technology (France) in accordance with the manufacturer's protocol. Frozen sperm doses were stored in liquid nitrogen at  $-196$  °C. After testing for biological usefulness and bacterial safety, at the end of the 28-day quarantine period, they were used for insemination (farms in the Sverdlovsk Province, 2019-2021).

Before insemination, the paillette was thawed for 10 s at  $+38$  °C in a water thermostat (PC Venera-Vet, Russia).

After heat synchronization, the cows were inseminated 2 times recto-cervically, introducing biomaterial into the cervix to a depth of 6-8 cm with a ShO-3 instrument (Russia).

Pregnancy was tested transrectally on day 45 after insemination by ultrasound scanning (a portable ultrasound scanning device Easi-scan, BCF Technology, Scotland). Under manual control, an ultrasound probe was inserted into the cow's rectum. At a frequency of 5 MHz, the active surface of the sensor was first directed to the bladder. Then the sensor was moved forward to the right to image the uterus. In pregnant cows, the uterine horns were slightly open, and the cotyledons of the placenta were visualized. From day 35 to day 45 of pregnancy, arcuate echopositive lines of the periembryonic membrane were detected around the embryo. Pregnancy was confirmed on day 60 by rectal examination to calculate

percentage of fertile inseminations.

The obtained data were processed statistically using the Microsoft Excel 2010 computer program. The tables show the means ( $M$ ) and errors of the means ( $\pm$ SEM). Statistical significance of differences was assessed by Student's  $t$ -test at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  significance levels.

**Results.** All physiological processes are subject to biorhythms which are genetically predetermined and regulated neurohumorally [25, 42]. The search for reliable and accessible indicators of biorhythms and the physiological state of the body remains urgent [64]. The main stress hormones are corticosteroids, in particular cortisol, with a circadian rhythm of synthesis and a peak concentration from 6 am to 8 am. Only free cortisol, which constitutes less than 10% of its total amount, has biological activity [65]. Cortisol levels can be measured in a variety of body fluids, tissues, and even excrement. It is believed that the content of cortisol in saliva correlates with a similar indicator for free cortisol in blood plasma and serum. Quantitative hair cortisol testing is a method for retrospectively assessing chronic stress [66].

For our purposes (determining cortisol peaks in stud bulls within 30 min after ejaculation), in our previous experience, venous blood serum is the most suitable biological fluid, despite the stress caused by sampling.

Table 1 shows the variability of free cortisol concentrations in all sires in the experiment.

**1. Individual variability of blood cortisol concentration (nmol/l) in 11 Holstein bulls (*Bos taurus*) of different ages ( $M \pm$ SEM, holding company GCV JSC, subsidiary breeding enterprise Uralplemtsentr JSC, Sverdlovsk Province, 2018)**

Index	Age, months										
	29	30	30	30	34	36	49	50	67	67	71
1	91.5 $\pm$ 19.3	74.8 $\pm$ 10.3	149.4 $\pm$ 21.9	55.1 $\pm$ 10.1	166.1 $\pm$ 29.4	83.7 $\pm$ 9.4	206.6 $\pm$ 27.3	167.8 $\pm$ 16.8	138.8 $\pm$ 31.9	210.8 $\pm$ 15.1	253.2 $\pm$ 20.3
2	42	34.3	70.8	28	71.1	49.5	129.4	107.3	101.1	160.7	152.3
3	197.8	118.3	253.4	105.0	333.3	126.8	329.6	229.7	183.4	299.5	339

Note. 1 — average of 7-8 samples, 2 and 3 — minimum and maximum cortisol concentrations, nmol/l, respectively.

To determine the daily dynamics of the endogenous hormones during the experiment, a total of 7-8 blood samples were collected from each bull. Table 1 shows that the cortisol concentration has no direct linear relationship with the age of the sire and is rather determined by the individual features of the organism, however, with age, the concentration of the hormone increases. Thus, in young animals ( $n = 6$ ) aged 29-36 months, the level of free cortisol varied from 28.0 to 333.3 nmol/l with average values from 55.1 $\pm$ 10.1 to 149.4 $\pm$ 21.9 nmol/l. In bulls older than 4 years (49-71 months), the cortisol concentration was approximately 2 times higher than in young bulls, from 138.8 $\pm$ 31.9 to 253.2 $\pm$ 20.3 nmol/l on average with a variation of 101.1 to 339 nmol/l.

**2. Blood cortisol concentration (nmol/l) in Holstein sires on the dates of semen collection (2/w technology) depending on age and stress duration ( $n = 11$ ,  $M \pm$ SEM, holding company GCV JSC, subsidiary breeding enterprise Uralplemtsentr JSC, Sverdlovsk Province, 2018)**

Group	Average age, months	1 <sup>st</sup> semen collection (control)	2 <sup>nd</sup> semen collection; 4-5-day stress	3 <sup>rd</sup> semen collection, 7-8-day stress
I ( $n = 6$ )	31.6 $\pm$ 1.1	70.8 $\pm$ 14.6	91.6 $\pm$ 19.5	144.7 $\pm$ 38.1
II ( $n = 5$ )	60.8 $\pm$ 4.7	153.1 $\pm$ 20.7*	172.5 $\pm$ 20.5*	163.8 $\pm$ 36.5*

Note. Group I is young bulls 29-36 months of age, group II is bulls over 4 years old. \* Differences from group I are statistically significant at  $p < 0.05$ .

Taking blood for animals is a kind of emotional and painful stress. In our tests, such daily manipulations for 2 weeks can be interpreted as prolonged habitual stress. During this period, semen was taken from the sires three times according

to the established schedule. Table 2 shows the average values of blood cortisol concentration on the dates of semen collection from sires of the younger and older age groups. As can be seen, initially and at all stages of the experiment, the concentration of endogenous cortisol in the older age group was significantly higher ( $p < 0.05$ ) than in the younger age group.

Emotional-painful stress on days 4-5 of veterinary blood collection procedures caused an increase in cortisol concentration in young animals by 23%, in adult animals by 11.3%, which is characterized as a stage of mobilization of the adaptive capabilities. At this stage, the hypothalamic-pituitary-adrenal system is activated, the blood level of cortisol increases, and catabolic processes begin to predominate for increased energy supply to the nervous system [1, 29, 67]. In young animals, further daily blood sampling led to an increase in the cortisol concentration by 51%, up to 144.7 nmol/l vs. 70.8 nmol/l at the beginning of the experiment. In adult bulls, cortisol levels decreased slightly, by 4.8% compared to the 2nd sampling.

In the group of young animals, the continued effect of the stressor forms adaptive mechanisms, energy consumption becomes less [8], but the amount of hormones produced, including cortisol, remains elevated. For adult animals it can be concluded that their adaptive capabilities have already been exhausted. Decreased cortisol levels confirm adrenal exhaustion at this stage [68]. At the first semen and blood collection the difference in the concentration of endogenous cortisol between young and adult sires at the beginning of the experiment was 54% ( $p < 0.05$ ). After the second semen collection, on days 4-5 of blood sampling stress, these differences remained statistically significant and amounted to 47% ( $p < 0.05$ ), by the third, final semen collection on days 7-8 with continuous daily veterinary manipulations, the difference between the groups reduced to 12% ( $p < 0.05$ ).

Blood samples from 11 stud bulls, 96 in total, were distributed in two groups depending on the age of the donors, 31.6 and 60.8 months on average, 47 39 samples, respectively (Table 3). Table 3 clearly demonstrates the percentage differences between groups for cortisol concentration ranks. The minimum cortisol concentration was 28 nmol/l for young animals (group I) and 129.4 nmol/l for bulls over 4 years old (group II). It was found that in 62% of serum samples from young stud bulls, the cortisol concentration did not exceed 100 nmol/l, and only for 38% of samples it ranged from 101 to 200 nmol/l. During the experiment, for 2 weeks, cortisol levels above 201 nmol/l were not recorded in young animals, while in 44% of blood serum samples from adult sires, its concentration exceeded 201.0 nmol/l.

**3. Distribution (%) of 96 blood serum samples by cortisol concentration on the date of semen collection in Holstein sires of different ages ( $n = 11$ ,  $M \pm SEM$ , holding company GCV JSC, subsidiary breeding enterprise Uralplemstsentr JSC, Sverdlovsk Province, 2018)**

Group	Cortisol concentration, nmol/l					
	up to 50	51-100	101-150	151-200	201-250	251 and more
I ( $n = 6$ )	21.3	40.4	19.2	19.2	0	0
II ( $n = 5$ )	0	0	30.8	25.6	23.1	20.6

Note. Group I is young bulls 29-36 months of age, group II is bulls over 4 years old.

We compared the average cortisol concentrations on the date of semen collection with the number of sperm in the ejaculate and the number of discarded ejaculates in young and adult animals for the entire experiment (Table 4).

The largest number of ejaculates that did not meet the requirements of GOST 23745-2014 for activity ( $60.0 \pm 10.9\%$  of the total) were obtained at the beginning of the experiment in the sires older than 4 years (see Table 4). The cortisol level and the number of sperm in the ejaculate of these animals was minimal and amounted to  $153.1 \pm 20.7$  nmol/l and  $6.6 \pm 1.3$  billion, respectively. In

young bulls, on the contrary, the largest number of low-quality ejaculates (58%) due to asthenospermia were registered on the last day of the experiment with a maximum number of sperm in the ejaculate of  $6.2 \pm 0.9$  billion and a cortisol level of  $144.7 \pm 38.1$  nmol/l, or 2 times higher than the initial one. The difference between the groups at this date was statistically significant ( $p < 0.05$ ).

**4. Blood cortisol concentration on the date of semen collection and the quality of ejaculates in Holstein sires of different ages during the experiment ( $n = 11$ ,  $M \pm SEM$ , holding company GCV JSC, subsidiary breeding enterprise Uralplemstsentr JSC, Sverdlovsk Province, 2018)**

Group	1 <sup>st</sup> semen collection (control)	2 <sup>nd</sup> semen collection; 4-5-day stress	3 <sup>rd</sup> semen collection, 7-8-day stress
Post-ejaculation cortisol concentration, nmol/l:			
I ( $n = 6$ )	$70.8 \pm 14.6$	$91.6 \pm 19.5$	$144.7 \pm 38.1$
II ( $n = 5$ )	$153.1 \pm 20.7^*$	$172.5 \pm 20.7^*$	$163.8 \pm 36.5^*$
Number of sperm per ejaculate, $\times 10^9$ :			
I ( $n = 6$ )	$4.0 \pm 0.7$	$5.5 \pm 0.5$	$6.2 \pm 0.9$
II ( $n = 5$ )	$6.6 \pm 1.3$	$7.5 \pm 1.3$	$7.3 \pm 1.2$
Rejected ejaculates, %:			
I ( $n = 6$ )	$25.0 \pm 8.8$	$16.7 \pm 7.6$	$58.3 \pm 10.1$
II ( $n = 5$ )	$60.0 \pm 10.9^*$	$30.0 \pm 10.25$	$20.0 \pm 8.9^*$

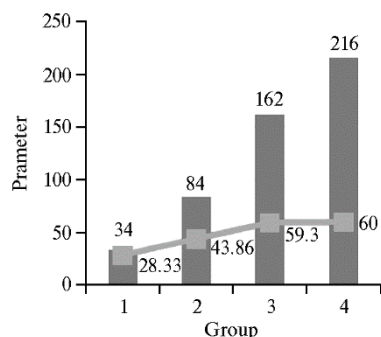
*N o t e.* Group I is young bulls 29-36 months of age, group II is bulls over 4 years old.  
\* Differences between groups are statistically significant at  $p < 0.05$ .

The minimum number of discarded ejaculates in bulls over 4 years old occurred on the last day of the experiment ( $20.0 \pm 8.9\%$  of the total), when the cortisol concentration was 5% lower than the maximum. In young sires, the smallest number of rejected ejaculates was obtained during the second semen collection (on days 4-5 of stress), with the cortisol concentration  $91.6 \pm 19.5$  nmol/l which was 23% higher vs. the beginning of the experiment but did not reached the maximum values.

It is possible that in adult bulls, fixed conditioned sexual reflexes to semen collection overcome short-term stress from the manipulation of blood sampling, while in young animals fear and stress from daily veterinary manipulations prevailed.

There was a direct relationship between the number of ejaculated sperm and the blood cortisol concentration after ejaculation in both young and adult sires.

It was previously shown that after doublet semen sampling, the level of cortisol in sires significantly increases over the next 48 h, especially in older animals [19]. Similar increase in cortisol concentration after ejaculation in bulls and boars was reported by K.E. Borg et al. [69] and J.D. Bishop et al. [70].



**The effectiveness of artificial insemination of cows (pregnancy, %; graph) depending on the blood cortisol level in Holstein sires after ejaculation (nmol/l, diagram): 1, 2, 3, 4 — groups of bulls depending on the cortisol concentration (22-45, 61-107, 140-216 and 216 nmol/l, respectively) (farms of the Sverdlovsk Province, 2019-2020).**

Although it is known that arousal and ejaculation are largely regulated by the hormonal system, there is still insufficient knowledge about the effect of hormone levels during ejaculation on the fertilizing ability of sperm. In our experiment, ejaculates in which the qualitative and quantitative parameters of the semen met the requirements of GOST 26030-2015 were diluted, packed, frozen according to the national technology [63] and used for artificial insemination of cows on farms in the Sverdlovsk Province. We assessed

the effectiveness of insemination depending on the blood cortisol concentration of stud bulls after ejaculation (Fig.).

All sires were conditionally divided into four groups depending on the blood cortisol concentration after ejaculation. A total of 60 cows were covered with semen of young sires with a cortisol level of 22-45 nmol/l (group 1), only 28.3% of these cows were pregnant. In group 2 of sires with a hormone level of 61-107 nmol/l, the semen was used to cover 57 cows, 43.9% inseminations of which were successful ( $p < 0.01$  vs. group 1). Group 3 was the sires older than 60 months with a cortisol concentration from 140 to 216 nmol/l. Their semen was used to inseminate 87 cows with a success rate of 59.3% ( $p < 0.001$  vs. group 1). The difference between groups 1 and 2, 2 and 3 was 15.6 and 15.4%, respectively. In group 4, in a sire older than 60 months, the cortisol concentration after ejaculation was 216 nmol/l, and of 20 cows, 60% were successfully inseminated.

Note that the effectiveness of artificial insemination was directly proportional to the blood cortisol concentration after ejaculation. We believe that the quantitative and qualitative indicators of semen in sires are determined by both the genotype and individual metabolism and hormonal status [25]. Stimuli caused by the stress of sexual arousal, subsequent mounting and ejaculation transmit signals to the hypothalamus. The resulting corticotropin-releasing hormone is sent to the hypothalamic-pituitary portal system, and within a few minutes, a large amount of cortisol appears in the blood, which promotes the mobilization of energy and makes glucose more available for various metabolic functions. Perhaps a higher level of blood cortisol in stud bulls after semen collection indicates strong sexual arousal, which can be regarded as a positive emotion with simultaneous significant physical activity accompanying mounting and ejaculation. In this case, the ejaculates are the most balanced due to the harmonious combination of the secretions of the accessory sex glands, and therefore the most complete and rich in carbohydrates, which better provides the energy supply of sperm and, as a result, has a positive effect on the effectiveness of insemination. The findings are consistent with our previous experiments [25] and studies in which a positive relationship between cortisol and testosterone levels was established in men [57, 58]. Similar results were obtained in beef bulls [56], horses and donkeys [61, 62].

So, in young sires ( $n = 6$ ) under stress from daily blood sampling, the average cortisol concentration after ejaculation increased uniformly during the experiment, amounting to 70.8, 91.1 and 144.7 nmol/l on the days of three consecutive doublet semen collections 4-5 days apart. In bulls older than 4 years ( $n = 5$ ), after the second semen collection, the maximum cortisol level of 172.5 nmol/l was recorded, but by the third collection it decreased slightly, by 5%. In young bulls, in 61.7% of blood samples the cortisol concentration was 100 nmol/l and only in 19.2% up to 150 nmol/l. In sires older than 4 years, the cortisol concentration in 30.8% of blood samples ranged from 101 to 150 nmol/l, the remaining 69.2% of samples ranged from 151 to 251 nmol/l and higher. As cortisol concentration increases after ejaculation, the number of sperm per ejaculate increases. In young bulls in three consecutive doublet semen collections 4-5 days apart, it was 4.0, 5.5 and 6.2 billion sperm, respectively, in adult producers 6.6, 7.3 and 7.5 billion sperm. The proportion of ejaculates rejected due to asthenospermia is not directly related to the blood cortisol level after ejaculation. In young bulls, the minimum number of rejected ejaculates (16.7%) corresponded to an average cortisol level of  $91.6 \pm 19.5$  nmol/l, in older bulls (20.0%) to a cortisol concentration of  $163.8 \pm 36.5$  nmol/l. The effectiveness of insemination directly depends on the cortisol level after ejaculation. The maximum percentage of pregnancies (59.3-60%) was noted when using semen from bulls with a cortisol concentration of 162 nmol/l and higher. In our opinion, more studies are necessary for a better understanding the physiology of sexual reflexes and their influence on the quality and

quantitative parameters of the ejaculate, its balance in the secretions of the accessory sex glands and resistance to cryopreservation. It is possible that quantification of steroid hormones during and after ejaculation will explain the cause of asthenospermia in one of the ejaculates during doublet collection. This will allow adjustments to be made to protocols for the use of breeding bulls and reduce the number of ejaculates subject to culling.

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