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Sexual dimorphism in neuro-endocrine-immune complex in intact rats

O. I. PLYSKA¹ <https://orcid.org/0000-0001-7002-7637>,

A. A. VOROBIIENKO^{1,2} <https://orcid.org/0009-0008-4982-1551>

¹ Mykhajlo Dragomanov Ukrainian State University, Kyiv, Ukraine

² OO Bohomolets' Institute of Physiology of NAS of Ukraine, Kyiv, Ukraine

E-mail: plys2005@ukr.net,

a.a.vorobiienko@npu.edu.ua

Annotation. Introduction and aim. Sexual dimorphism across animals and human in nervous, endocrine and immune systems has been the subject of a number of studies, but each of them was limited to one system. The aim of this study is the simultaneous comparison of parameters of the autonomic, endocrine and immune systems, which closely interact with each other within the framework of the triune neuro-endocrine-immune complex. The scientific novelty of this work lies in the integrated, system-level analysis of all three regulatory systems simultaneously in the same animals, which distinguishes it from previous studies conducted by other researchers who examined individual systems in isolation.

Material and methods. The experiment was conducted on 20 intact rats Wistar line: 10 males and 10 females. We calculated the parameters of the HRV: Mode, Amplitude of the mode and Variational scope as markers of the circulating catecholamines, sympathetic and vagal tones respectively. Among endocrine parameters determined serum levels of main adaptation hormones such as Corticosterone, Aldosterone, Testosterone, Triiodothyronine, as well as Parathyroid hormone and Calcitonin. In addition, the thickness of the glomerular, fascicular, reticular and medullary zones of the adrenal glands was measured in smears under a microscope. The percentage of cells populations and the parameters of phagocytosis by neutrophils and monocytes of *Staphylococcus aureus* were determined in the blood. The Thymus and Spleen were weighed and made smears-imprints for counting Thymocytoqram and Splenocytoqram.

Results. It was established that males have significantly higher levels of 11 variables: serum testosterone and triiodothyronine, percentage of epitheliocytes, endotheliocytes, and macrophages in the thymocytoqram and its entropy, percentage of lymphocytes and lymphoblasts in the splenocytoqram, phagocytosis activity of blood microphages and macrophages, as well as percentage of 0-Lymphocytes. On the other hand, females have significantly higher levels of 19 variables: serum corticosterone, catecholamines, PTH, calcitonin, aldosterone, the mass of the adrenal glands and the thickness of their fascicular, reticular and glomerular zones, the percentage of lymphocytes and lymphoblasts in the thymocytoqram, fibroblasts in the splenocytoqram and its entropy, natural killers and B-Lymphocytes in the immunocytoqram of blood and its entropy, intensity of phagocytosis of macrophages as well as reaction of blast transformation of T-Lymphocytes to phytohemagglutinin. Using the method

of discriminant analysis, 14 of the listed variables were selected as specific endocrine-immune markers of sex.

Conclusion. Intact rats show pronounced sexual dimorphism in a number of indicators of the endocrine and immune systems, which can be assessed quantitatively by the Mahalanobis distance ($D^2 = 2620$), indicating a very large inter-group separation, and by the difference between the discriminant roots (51.2), reflecting the magnitude of between-sex variance explained by the selected variables. This approach will make it possible to quantitatively assess the influence of stressors and adaptogens on sexual endocrine-immune dimorphism in subsequent studies.

Key words: adaptation hormones, HRV, adrenal glands, thymus, spleen, immunocytes of blood, phagocytosis, male and female rats.

Статевий диморфізм у нейроендокриноімунному комплексі в інтактних щурів

О. І. ПЛИСКА¹, А. А. ВОРОБІШКО^{1, 2}

¹ Український державний університет імені Михайла Драгоманова, Київ, Україна

² Інститут фізіології імені О. О. Богомольця НАН України, Київ, Україна

E-mail: plys2005@ukr.net,

a.a.vorobiinko@npu.edu.ua

Резюме. Статевий диморфізм у тварин і людини в нервовій, ендокринній та імунній системах був предметом низки досліджень, але кожне з них обмежувалося однією системою. Метою цього дослідження є одночасне порівняння параметрів вегетативної, ендокринної та імунної систем, які тісно взаємодіють одна з одною в рамках триединого нейро-ендокринно-імунного комплексу. Наукова новизна цієї роботи полягає в інтегрованому, системному аналізі всіх трьох регуляторних систем одночасно в одних і тих самих тварин, що відрізняє її від попередніх досліджень, проведених іншими дослідниками, які досліджували окремі системи ізольовано.

Матеріали та методи. Експеримент було проведено на 20 інтактних щурах лінії Вістар: 10 самцях та 10 самках. Ми розраховували параметри ВСР: моду, амплітуду моди та варіаційний розмах як маркери циркулюючих катехоламінів і симпатичного та вагусного тонусів відповідно. Серед ендокринних параметрів визначали рівні в сироватці крові основних адаптаційних гормонів, таких як кортикостерон, альдостерон, тестостерон, трийодтиронін, а також паратиреоїдного гормону та кальцитоніну. Крім того, під мікроскопом вимірювали товщину клубочкової, фасцикулярної, ретикулярної та мозкової зон надниркових залоз у мазках-відбитках. У крові визначали відсоток популяцій лейкоцитів та параметри фагоцитозу нейтрофілами та моноцитами *Staphylococcus aureus*. Зважували тимус та селезінку та робили мазки-відбитки для підрахунку тимоцитограми та спленоцитограми.

Результати. Було встановлено, що у самців спостерігаються значно вищі рівні 11 змінних: тестостерону та трийодтироніну в сироватці крові, відсотка епітеліоцитів, ендотеліоцитів та макрофагів у тимоцитограми та її ентропії, відсотка лімфоцитів та лімфобластів у спленоцитограми, фагоцитарної активності мікрофагів та макрофагів крові, а також відсотка 0-лімфоцитів. З іншого боку, у самок спостерігаються значно вищі рівні 19 змінних: кортикостерону в сироватці крові, катехоламінів, ПТГ, кальцитоніну, альдостерону, маси надниркових залоз та товщини їх фасцикулярної, ретикулярної та клубочкової зон, відсотка лімфоцитів та лімфобластів у тимоцитограми, фібробластів у спленоцитограми та її ентропії, природних

кілерів та В-лімфоцитів в імуноцитогамі крові та її ентропії, інтенсивності фагоцитозу макрофагів, а також реакції бластної трансформації Т-лімфоцитів на фітогемаглютинін. За допомогою методу дискримінантного аналізу було відібрано 14 із перелічених змінних в якості специфічних ендокринно-іmunних маркерів статі.

Висновок. Інтактні щури демонструють виражений статевий диморфізм за низкою показників ендокринної та імунної систем, який можна кількісно оцінити за допомогою відстані Махаланобіса ($D^2 = 2620$), що вказує на дуже велику міжстатеву розділеність, та за різницею між дискримінантними коренями (51,2), що відображає величину міжстатеву дисперсії, що пояснюється обраними змінними. Такий підхід дозволить кількісно оцінити вплив стресорів та адаптогенів на статевий ендокринно-іmunний диморфізм у наступних дослідженнях.

Ключові слова: адаптаційні гормони, ВСР, надниркові залози, тимус, селезінка, імуноцити крові, фагоцитоз, самці та самиці щурів.

Introduction. Sexual dimorphism across animals and human in nervous, endocrine and immune systems as well as metabolism has been the subject of a number of studies, but each of them was limited to one system (Reznikov et al, 2001; 2004; Bjelobaba et al, 2015; Foo et al, 2017; Kelly et al, 2018; Jaillon et al, 2019; Joseph-Bravo et al, 2020; Song et al, 2021; Kiriakopoulos et al, 2022; Alexander et al, 2023; Zhang et al, 2024; Reznikov, 2026). The aim of this study is the simultaneous comparison of parameters of the autonomic, endocrine and immune systems, which closely interact with each other within the framework of the triune neuro-endocrine-immune complex, as well as with parameters of metabolism (Bilas et al, 2020; Popovych et al, 2020; 2022; Kozyavkina et al, 2021; Gozhenko et al, 2023; Plyska et al, 2023; Korda et al, 2025).

The scientific novelty of the present work consists in the integrated, simultaneous assessment of all three regulatory systems – autonomic, endocrine, and immune – within the same experimental animals, combined with the application of Shannon entropy analysis and multivariate discriminant statistics. This approach distinguishes the present study from previous work (Popovych et al, 2018; 2020), in which a similar design was used but the scope of immune parameters was more limited, and from independent international studies that examined individual systems in isolation (Jaillon et al, 2019; Song et al, 2021; Zhang et al, 2024). The simultaneous characterisation of all three systems in the same animals allows detection of inter-system correlations and integrated endocrine-immune profiles that cannot be identified when systems are studied separately.

Material and methods. Ethics approval. All animals were kept in room having temperature $22 \pm 2^\circ\text{C}$, and relative humidity of 44–55 % under 12/12 hours light and dark cycle with standard laboratory diet and water given ad libitum. Studies have been conducted in accordance with the rules and requirements of the «General Principles for the Work on Animals» approved by the I National Congress on Bioethics (Kyiv, Ukraine, 2001) and agreed with the provisions of the «European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes» (Council of Europe No 123, Strasbourg 1985), and the Law of Ukraine «On the Protection of Animals from Cruelty» of 26.02.2006. The removal of animals from the experiment was carried out under light inhalation (ether) anesthesia by decapitation. The conduct of experiments was approved by the Ethics Committee of the Bohomolets' Institute of Physiology.

Study design and procedure. The present article reports data from intact rats only – 10 males and 10 females – drawn from a larger experiment comprising five groups of both sexes ($n = 100$ total). The full experimental design, including groups exposed to chronic aversive stress and treated with adaptogens, is described elsewhere (Fihura et al, 2023; Zukow et al, 2024; 2024a;

Korda *et al.*, 2025); the present analysis is restricted to the intact control group in order to characterise baseline sexual dimorphism before any experimental intervention.

Over the 10 days, one male and female rat Wistar line remained intact and 4 other pairs were exposed to chronic aversive stress (Reznikov *et al.*, 2001; Popovych *et al.*, 2020) for 6 days by placing them in individual tight Plexiglas chambers for 30 min, which was preceded by injecting daily water (3 mL/200g) with a syringe through a curved needle with smooth olive into the esophagus. In other two groups, the classical adaptogen ginseng (alcohol tincture produced by Vishpha®) in a dose of 0.01 mL/200g or the Ukrainian phytocomposition «Balm Truskavets» (produced by PRPE «Ukrainian Balms», Mykolaiv, Ukraine; TV Y 15.8-24055046-005:2009) in a dose of 0.06 mL/200g was added to the water (Fihura *et al.*, 2023; Zukow *et al.*, 2024; 2024a; Korda *et al.*, 2025).

The day after completion the course of immobilization and water loads, ECG under light ether anesthesia was recorded, by inserting needle electrodes under the skin of the paws. Right away the animals removed from the experiment by decapitation in order to remove the adrenal glands, thymus and spleen, as well as collect the maximum possible amount of blood in which was determined some endocrine and immune parameters.

Based on about 120 R-R intervals we calculated the parameters of the HRV: Mode (Mo), Amplitude of the mode (AMo) and Variational scope (MxDMn) as markers of the circulating Catecholamines, Sympathetic and Vagal tones, respectively (Baevsky & Chernikova, 2017).

Among endocrine parameters determined serum levels of main adaptation hormones such as Corticosterone, Aldosterone, Testosterone, Triiodothyronine, as well as Parathyroid hormone and Calcitonin (by ELISA, with the use of analyzer «RT-2100C» and corresponding sets of reagents from «Alkor Bio», XEMA Co, Ltd and DRG International Inc).

In addition, the thickness of the glomerular, fascicular, reticular and medullary zones of the adrenal glands was measured in smears under a microscope (Popovych *et al.*, 2022).

Among the immune parameters of the blood, first of all, analysis of Leukocytogram (LCG), in the percentage of lymphocytes (L), monocytes (M), eosinophils (Eo), basophils (Bas), rod-shaped (RN) and polymorphonuclear (PMNN) neutrophils was performed. Based on these data, the Entropy of the Leukocytogram (hLCG) was calculated according to the equation derived by Popovych (Popovych, 2011; Popovych *et al.*, 2020; Bilas *et al.*, 2020; Gozhenko *et al.*, 2021) on the basis of the classical Shannon's (1948) equation:

$$hLCG = - (L \cdot \log_2 L + M \cdot \log_2 M + Eo \cdot \log_2 Eo + Bas \cdot \log_2 Bas + \\ + RN \cdot \log_2 RN + PMNN \cdot \log_2 PMNN) / \log_2 6$$

The percentage of theophylline-resistant (TR) and theophylline-susceptible (TS) T-lymphocytes, B-lymphocytes, plasma cells (Pla), natural killers (NK), and 0-lymphocytes were identified, as described in the manual (Perederiy *et al.*, 1995).

For these components the Entropy of the Immunocytogram (hICG) also was calculated by Popovych's equation:

$$hICG = - (TR \cdot \log_2 TR + TS \cdot \log_2 TS + B \cdot \log_2 B + Pla \cdot \log_2 Pla + \\ + NK \cdot \log_2 NK + 0L \cdot \log_2 0L) / \log_2 6$$

In addition, we tested the reaction of blast transformation of T-Lymphocytes to phytohemagglutinin (Perederiy *et al.*, 1995).

About the condition of the phagocytic function of neutrophils (microphages) and monocytes (macrophages) were judged by the phagocytosis index (PhI, percentage of cells, in which found microbes), the microbial count (MC, number of microbes absorbed by one phagocyte) and the killing index (KI, percentage of dead microbes) for *Staphylococcus aureus* (ATCC N25423 F49). Based on these parameters, taking into account the absolute content of neutrophils (N)

and monocytes (M), their bactericidal capacity (BCC N&M) was calculated by equation (Popovych *et al*, 2022):

$$\text{BCC N\&M} = \text{N\&M} \cdot \text{PhI} \cdot \text{MC} \cdot \text{KI}$$

The Thymus and Spleen were weighed and made smears-imprints for counting Thymocytoqram and Splenocytoqram (Horizontov *et al*, 1983; Bilas *et al*, 2020). The components of the Thymocytoqram (TCG) are lymphocytes (Lc), lymphoblastes (Lb), reticulocytes (Ret), macrophages (Mac), endotheliocytes (En), epitheliocytes (Ep), and Hassal's corpuscles (H). The Splenocytoqram (SCG) includes lymphocytes (Lc), lymphoblastes (Lb), plasma cells (Pla), reticulocytes (R), macrophages (Ma), fibroblasts (F), microphages (Mi), and eosinophils (Eo).

For them Shannon's Entropy was calculated too:

$$\text{hTCG} = -(\text{Lc} \cdot \log_2 \text{Lc} + \text{Lb} \cdot \log_2 \text{Lb} + \text{Ret} \cdot \log_2 \text{Ret} + \text{Mac} \cdot \log_2 \text{Mac} + \text{En} \cdot \log_2 \text{En} + \text{Ep} \cdot \log_2 \text{Ep} + \text{H} \cdot \log_2 \text{H}) / \log_2 7;$$

$$\text{hSCG} = -(\text{Lc} \cdot \log_2 \text{Lc} + \text{Lb} \cdot \log_2 \text{Lb} + \text{Pla} \cdot \log_2 \text{Pla} + \text{R} \cdot \log_2 \text{R} + \text{Ma} \cdot \log_2 \text{Ma} + \text{F} \cdot \log_2 \text{F} + \text{Mi} \cdot \log_2 \text{Mi} + \text{Eo} \cdot \log_2 \text{Eo}) / \log_2 8$$

Statistical analysis. Statistical processing was performed using Microsoft Excel and Statistica 6.4 (StatSoft Inc., Tulsa, OK, USA). Group differences were assessed using Student's independent-samples t-test. Given the sample size of $n = 10$ per group, which represents the minimum recommended for parametric testing, a post-hoc statistical power analysis was performed: for the largest observed effect (testosterone, Z-score difference = 1.78, SD pooled ≈ 0.14), power exceeded 0.99; for the smallest significant effect (macrophages in TCG, $t = 3.79$, $df = 18$), power was approximately 0.87, which is above the conventional threshold of 0.80. These calculations confirm that the sample size was adequate for detecting the reported effects. For variables that did not meet the assumption of normality (Shapiro–Wilk test), results were additionally verified using the Mann–Whitney U test; conclusions were consistent in all cases.

Actual values of variables were normalised by Z-score transformation: $Z = (V - M) / \text{SD}$, where V is the actual value, M is the common mean for males and females combined, and SD is the common standard deviation. Z-score normalisation enables comparison of diverse variables on a uniform scale (Polovynko *et al*, 2016). Discriminant analysis was performed to identify the subset of variables that best discriminated between sexes; the Mahalanobis distance (D^2) and the difference between discriminant roots were used as quantitative measures of inter-group separation.

Results and discussion. In this article will be characterized only intact rats: 10 males (Weight Mean = 256 g; SD = 32 g) and 10 females (Mean = 262 g; SD = 28 g).

Neuro-Endocrine Profile. The profile of neuro-endocrine variables (Table 1 and Fig. 1) shows that males have an order-of-magnitude higher level of testosterone by definition, as well as a significantly higher level of triiodothyronine. The higher testosterone level in males reflects the dominant role of the testes as the primary androgenic organ, in contrast to females in whom adrenal androgens (reticular zone) represent the main source (Burger, 2002). The higher T3 level in males is consistent with the known stimulatory effect of testosterone on thyroid hormone synthesis and peripheral conversion of T4 to T3 (Joseph-Bravo *et al*, 2020).

On the other hand, females have significantly higher levels of aldosterone and corticosterone, as well as greater thickness of their respective adrenocortical sources – the glomerular and fascicular zones – as well as the reticular zone, which serves as an additional source of androgens (Burger, 2002). In addition, despite comparable thickness of the medullary zone and comparable sympathetic tone (AMo), females show higher levels of circulating catecholamines (reflected by higher Mo^{-1}), as well as higher calcitonin and PTH. The higher adrenocortical activity in females is consistent with the well-documented stim-

ulatory effect of oestrogens on the hypothalamic-pituitary-adrenal axis (*Kiriakopoulos et al*, 2022). The higher circulating catecholamine level in females, in the absence of a corresponding difference in sympathetic tone, suggests a greater adrenomedullary secretory response to equivalent autonomic drive, possibly mediated by oestrogen-dependent upregulation of phenylethanolamine N-methyltransferase (PNMT) activity.

Table 1

Sexual dimorphism in endocrine and autonomic parameters in intact rat

Sex	Males (10)	Females (10)	Males (10)	Females (10)	Student's statistics	
Variables	Raw values		Z-scores		Cv	t
Testosterone, nM/L	32,0 ± 3,0	4,49 ± 0,51	0,89 ± 0,19	−0,89 ± 0,03	0,850	8,97d
Triiodothyronine, nM/L	2,65 ± 0,12	2,07 ± 0,17	0,53 ± 0,22	−0,53 ± 0,32	0,231	2,73b
AMo as sympathetic tone, %	54 ± 8	54 ± 5	−0,01 ± 0,39	0,01 ± 0,24	0,373	0,04
Medullary zone AG, μM	86 ± 7	92 ± 10	−0,11 ± 0,27	0,11 ± 0,37	0,307	0,48
MxDmN as vagal tone, msec	44 ± 8	53 ± 13	−0,14 ± 0,25	0,14 ± 0,38	0,697	0,63
Aldosterone, pM/L	552 ± 32	735 ± 75	−0,46 ± 0,16	0,46 ± 0,37	0,312	2,25a
Calcitonin, ng/L	20,7 ± 1,0	35,6 ± 5,9	−0,49 ± 0,06	0,49 ± 0,39	0,532	2,49a
Parathyroid hormone, μg/L	139 ± 5	200 ± 21	−0,54 ± 0,09	0,54 ± 0,37	0,333	2,83b
Glomerular zone AG, μM	129 ± 11	193 ± 9	−0,70 ± 0,25	0,70 ± 0,20	0,282	4,38d
Mode ^{−1} as catecholamines, ms ^{−1}	186 ^{−1} ± 12 ^{−1}	125 ^{−1} ± 4 ^{−1}	−0,76 ± 0,27	0,76 ± 0,11	0,272	4,75d
Adrenal glands mass, mg	50,2 ± 1,3	67,0 ± 2,8	−0,77 ± 0,12	0,77 ± 0,26	0,186	5,77d
Reticular zone AG, μM	20,0 ± 1,8	45,7 ± 3,7	−0,80 ± 0,11	0,80 ± 0,23	0,487	6,20d
Fascicular zone AG, μM	223 ± 12	375 ± 18	−0,83 ± 0,13	0,82 ± 0,20	0,306	7,03d
Corticosterone, nM/L	293 ± 14	778 ± 56	−0,87 ± 0,05	0,87 ± 0,20	0,521	8,41d

Note: p < 0,05a; p < 0,02b; p < 0,01c; p < 0,001.

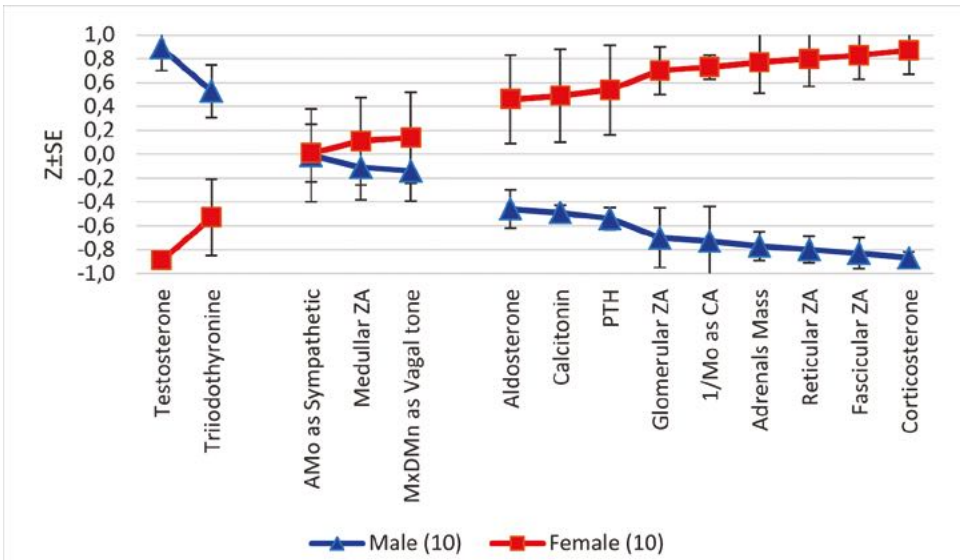


Fig. 1. Profile of neuro-endocrine variables in intact rats

The results of this study confirm, complement, and refine the results of a previous study conducted with a similar design on 20 intact rats (*Popovych et al*, 2018; 2020). The most significant sex differences were found to be related to the morpho-functional parameters of the adrenal glands. In particular, females have higher androgenic, glucocorticoid, and mineralocorticoid activity, estimated according to the thickness of the reticular zone of the adrenal cortex and daily excretion in the urine of 17-ketosteroids, the thickness of the fascicular corticoadrenal zone, and the plasma level of corticosterone as well as of the thickness of glomerular zone of the adrenal cortex as well as plasma and urine sodium and potassium. However, females have higher parathyroid and calcitonin activities, as measured by plasma and urinary levels of calcium and phosphate. In addition, females have a smaller amount of HRV mode as an inverse measure of heart rhythm, which is subject to the so-called humoral regulation channel (circulating catecholamines, glucocorticoids, glucagon, electrolytes, etc.), whereas difference in HRV-markers of sympathetic and vagal tone was not significant. Instead, the plasma triiodothyronine level was 85 ± 15 % of the male level. Regarding calcitonin, a discrepancy was noted between the present findings – higher calcitonin in females – and data reported by *Fihura et al* (2024) and *Korda et al* (2025), who found higher calcitonin levels in male rats. Possible explanations include differences in the season of experimentation (calcitonin secretion shows circannual variation), differences in the age and weight of animals, or methodological differences in the ELISA kits used. This discrepancy warrants further investigation.

Thymocytoqram. For the same thymus mass and percentage of reticulocytes and Hassal’s corpuscles in the thymocytoqram, the entropy of the latter and the percentage of epitheliocytes, endotheliocytes, and macrophages were found to be higher in males, while the percentage of lymphoblasts and T-lymphocytes was lower than in females (Table 2 and Fig. 2). The higher entropy of the thymocytoqram in males, despite lower lymphocyte content, reflects a more diverse cellular composition of the thymic microenvironment, with proportionally greater representation of stromal cells (epitheliocytes, endotheliocytes) and macrophages. This pattern may be related to the known immunosuppressive effects of testosterone on T-lymphocyte proliferation and thymic output (*Foo et al*, 2017), resulting in relative enrichment of non-lymphoid populations.

Table 2

Sexual dimorphism in thymus and thymocytoqram parameters in intact rats

Sex	Males (10)	Females (10)	Males (10)	Females (10)	Student's statistics	
Variables	Raw values		Z-scores		Cv	t
Thymocytoqram Entropy, •103	561 ± 8	423 ± 10	0,91 ± 0,11	–0,91 ± 0,13	0,154	10,8d
Epitheliocytes, %	19,8 ± 0,7	8,5 ± 0,6	0,92 ± 0,11	–0,92 ± 0,10	0,435	12,0d
Endotheliocytes, %	7,20 ± 0,49	2,60 ± 0,31	0,86 ± 0,18	–0,86 ± 0,11	0,546	7,97d
Macrophages, %	4,70 ± 0,21	3,00 ± 0,39	0,65 ± 0,16	–0,65 ± 0,30	0,340	3,79c
Thymus mass index, mg/100g	29 ± 2	25 ± 2	0,32 ± 0,32	–0,31 ± 0,29	0,249	1,39
Thymus mass, mg	76 ± 5	66 ± 5	0,30 ± 0,31	–0,30 ± 0,30	0,226	1,39
Reticulocytes, %	5,40 ± 0,54	4,60 ± 0,54	0,23 ± 0,32	–0,23 ± 0,32	0,343	1,04
Hassal's corpuscles, %	1,70 ± 0,27	1,85 ± 0,17	–0,11 ± 0,39	0,11 ± 0,24	0,393	0,47
Lymphoblastes, %	5,40 ± 0,16	7,50 ± 0,27	–0,82 ± 0,13	0,82 ± 0,21	0,198	6,68d
Lymphocytes, %	55,8±1,1	70,1 ± 0,7	–0,91 ± 0,13	0,91 ± 0,10	0,124	11,1d

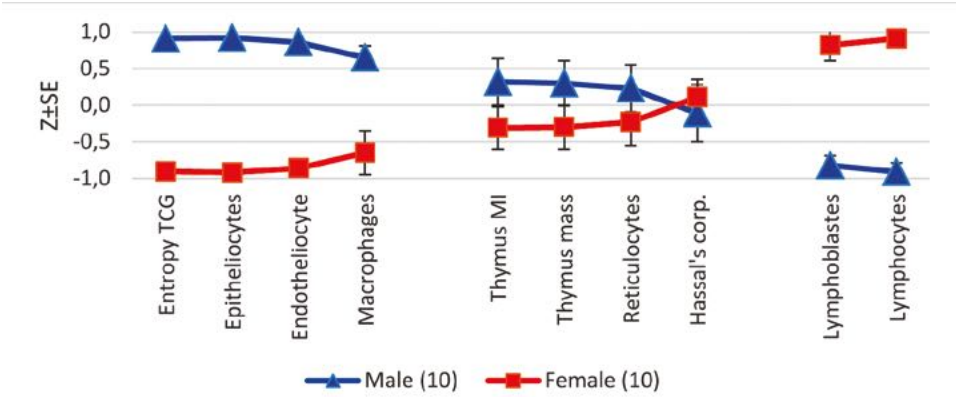


Fig. 2. Profile of Thymocytoqram variables in intact rats

Compared to previous ones, our data are consistent with respect to all variables (Popovych *et al*, 2018; 2020), but with others (Fihura *et al*, 2024; Korda *et al*, 2025) only with respect to 4 variables: mass, entropy, lymphocytes and Hassal’s corpuscles.

Splenocytoqram. The mass of the spleen was the same in both sexes, as was the percentage of eosinophils, plasma cells, and reticulocytes (Table 3 and Fig. 3). At the same time, a higher percentage of lymphocytes and lymphoblasts was found in males, but a lower percentage of microphages, macrophages, and fibroblasts, as well as lower entropy of the splenocytoqram. The lower splenocytoqram entropy in males reflects a less diverse cellular composition of the splenic parenchyma, dominated by lymphoid cells, in contrast to females in whom a broader representation of myeloid and stromal elements (macrophages, fibroblasts) contributes to higher entropy. This pattern is consistent with the known pro-inflammatory and phagocyte-activating effects of oestrogens (Jaillon *et al*, 2019; Song *et al*, 2021).

Table 3

Sexual dimorphism in spleen and splenocytoqram parameters in intact rats

Sex	Males (10)	Females (10)	Males (10)	Females (10)	Student's statistics	
Variables	Raw values		Z-scores		Cv	t
Lymphocytes, %	53,3 ± 0,9	48,7 ± 0,9	0,64 ± 0,25	−0,64 ± 0,24	0,071	3,69 ^c
Lymphoblastes, %	5,20 ± 0,36	3,80 ± 0,36	0,53 ± 0,27	−0,53 ± 0,27	0,293	2,76 ^b
Eosinophiles, %	1,80 ± 0,25	1,71 ± 0,29	0,05 ± 0,30	−0,05 ± 0,35	0,479	0,23
Plasmocytes, %	2,50 ± 0,48	2,40 ± 0,52	0,03 ± 0,31	−0,03 ± 0,34	0,628	0,14
Spleen mass index, mg/100g	285 ± 12	308 ± 33	−0,15 ± 0,16	0,15 ± 0,42	0,259	0,68
Spleen mass, mg	749 ± 43	818 ± 82	−0,17 ± 0,21	0,17 ± 0,40	0,262	0,74
Reticulocytes, %	14,3 ± 0,6	14,3 ± 0,6	0,00 ± 0,32	0,00 ± 0,34	0,124	0,00
Microphages, %	11,6 ± 0,5	12,9 ± 0,5	−0,40 ± 0,31	0,40 ± 0,28	0,132	1,92
Macrophages, %	5,70 ± 0,70	7,90 ± 0,50	−0,50 ± 0,32	0,50 ± 0,23	0,322	2,55 ^b
Fibroblastes, %	6,00 ± 0,42	8,20 ± 0,66	−0,54 ± 0,21	0,54 ± 0,32	0,289	2,80 ^b
Splenocytoqram Entropy, •10 ³	719 ± 9	754 ± 9	−0,54 ± 0,26	0,54 ± 0,27	0,044	2,87 ^c

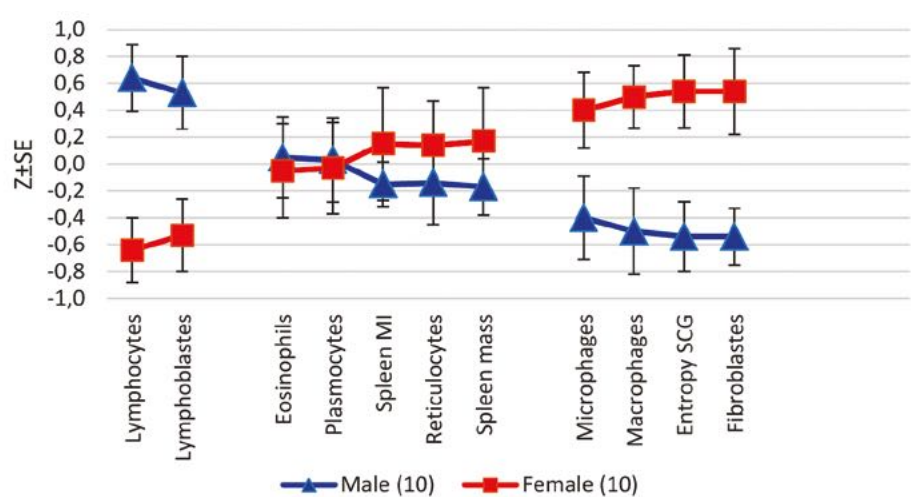


Fig. 3. Profile of Splenocytogram variables in intact rats

Regarding the mass, plasma cells and macrophages, our data are consistent with those obtained in the previous study (Fihura et al, 2024; Korda et al, 2025), instead, the authors found a higher percentage of eosinophils and a lower percentage of reticulocytes in males.

Comparative analysis of the content of lymphocyte populations in the blood (Table 4 and Fig. 4) revealed a significant advantage of females in relation to B-Lymphocytes and natural killers as well as reaction of blast transformation of T-Lymphocytes to phytohemagglutinin and Immunocytogram Entropy. This is combined with a lower content of O-lymphocytes. Instead, the percentage of theophylline-resistant and theophylline-susceptible T-lymphocytes as well as Plasmocytes did not differ significantly in both sexes.

Our data are consistent with those obtained by Popovych et al (2018; 2020), on the other hand, in another study (Fihura et al, 2024; Korda et al, 2025) in females a lower content of B-Lymphocytes and a higher content of Plasmocytes were found in the absence of differences regarding the remaining variables.

Leukocytogram. The leukocytogram did not reveal statistically significant differences between males and females in the percentage of lymphocytes, monocytes, eosinophils, basophils, rod-shaped neutrophils, or polymorphonuclear neutrophils, nor in the entropy of the leukocytogram (Table 4 and Fig. 4). This finding indicates that the peripheral blood differential leukocyte count is not a sensitive marker of sexual dimorphism under intact (non-stressed) conditions, in contrast to the more informative immunocytogram parameters described below.

Table 4

Sexual dimorphism in blood leukocytes populations in intact rats

Sex	Males (10)	Females (10)	Males (10)	Females (10)	Student's statistics	
Variables	Raw values		Z-scores		Cv	t
PMN neutrophils, %	28,1 ± 1,7	26,8 ± 2,2	0,11 ± 0,27	-0,11 ± 0,37	0,224	0,46
Pan lymphocytes, %	60,5 ± 1,4	59,7 ± 2,6	0,06 ± 0,22	-0,06 ± 0,40	0,109	0,27
Basophils, %	0,28 ± 0,12	0,28 ± 0,12	0,00 ± 0,32	0,00 ± 0,32	1,319	0,00
Rod-shaped neutrophils, %	3,50 ± 0,17	3,50 ± 0,40	0,00 ± 0,18	0,00 ± 0,42	0,270	0,00
Eosinophils, %	3,60 ± 0,60	4,30 ± 0,68	-0,17 ± 0,30	0,17 ± 0,34	0,509	0,77
Monocytes, %	4,10 ± 0,69	5,02 ± 0,84	-0,19 ± 0,29	0,19 ± 0,35	0,530	0,85
Leukocytogram Entropy, •103	537 ± 11	591 ± 21	-0,17 ± 0,21	0,17 ± 0,40	0,091	0,75

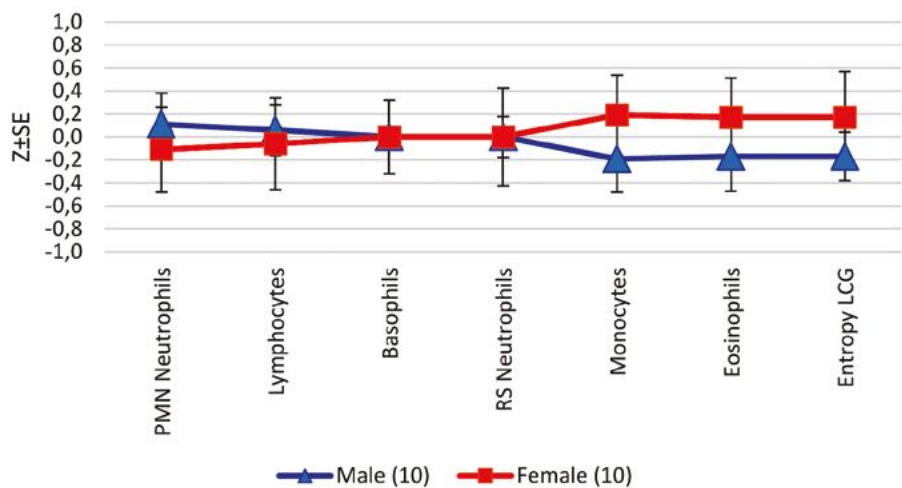


Fig. 4. Profile of Leukocytogram variables in intact rats

Blood Immunocytogram. In contrast to the leukocytogram, the immunocytogram revealed pronounced sexual dimorphism (Table 5 and Fig. 5). Higher percentages of natural killers and B-lymphocytes, as well as higher entropy of the immunocytogram, were found in females, whereas males showed higher percentages of 0-lymphocytes. The higher NK and B-lymphocyte counts in females are consistent with the well-established immunoenhancing effects of oestrogens on humoral and innate cytotoxic immunity (*Jaillon et al*, 2019; *Zhang et al*, 2024). The higher 0-lymphocyte percentage in males may reflect a larger pool of undifferentiated or non-committed lymphocytes, possibly related to testosterone-mediated suppression of lymphocyte maturation and differentiation.

Table 5

Sexual dimorphism in blood lymphocytes populations in intact rats

Sex	Males (10)	Females (10)	Males (10)	Females (10)	Student's statistics	
Variables	Raw values		Z-scores		Cv	t
0-Lymphocytes, %	29,6 ± 1,5	23,5 ± 1,4	0,57 ± 0,28	-0,57 ± 0,25	0,203	3,04c
TR T-helper Lymphocytes, %	32,2 ± 0,8	30,9 ± 0,6	0,29 ± 0,34	-0,29 ± 0,27	0,070	1,34
Plasmocytes, %	0,74 ± 0,26	0,49 ± 0,16	0,18 ± 0,39	-0,18 ± 0,23	1,098	0,82
TS T-cytolytic Lymphocytes, %	14,3 ± 0,9	15,2 ± 1,0	-0,16 ± 0,30	0,16 ± 0,34	0,194	0,69
B-Lymphocytes, %	12,8 ± 0,7	15,1 ± 0,7	-0,48 ± 0,29	0,48 ± 0,28	0,170	2,43b
Immunocytogram Entropy, • 10³	850 ± 9	878 ± 6	-0,50 ± 0,33	0,50 ± 0,22	0,032	2,52a
BTR of T-Lymphocytes, %	68,3 ± 3,3	83,6 ± 2,0	-0,66 ± 0,29	0,67 ± 0,17	0,152	3,97d
NK Lymphocytes, %	10,3 ± 0,6	14,8 ± 0,4	-0,80 ± 0,21	0,80 ± 0,16	0,222	6,10d

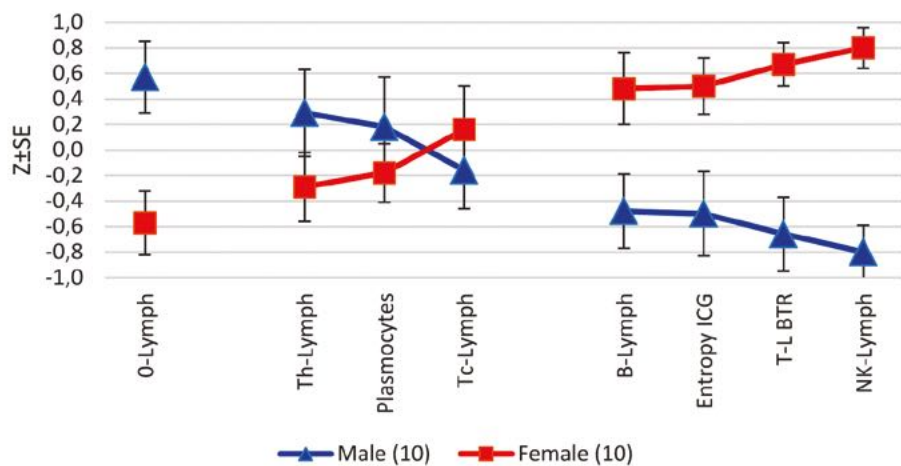


Fig. 5. Profile of Immunocytogram variables in intact rats

Phagocytosis. Higher phagocytic activity (phagocytosis index) of both neutrophils (microphages) and monocytes (macrophages) was found in males, whereas the intensity of phagocytosis (microbial count per phagocyte) of macrophages was higher in females. The reaction of blast transformation of T-lymphocytes to PHA was also higher in females (Table 6 and Fig. 6). The higher phagocytic activity in males, combined with lower phagocytic intensity per cell, suggests that testosterone promotes the recruitment of phagocytes to the site of infection (higher percentage of active cells) but does not enhance per-cell bactericidal capacity. In contrast, the higher macrophage phagocytic intensity in females may reflect oestrogen-mediated upregulation of Fc receptor expression and intracellular killing mechanisms (*Jaillon et al*, 2019). The higher RBTL-PHA in females indicates a stronger T-lymphocyte proliferative capacity, which is consistent with the known immunostimulatory effects of oestrogens on adaptive cellular immunity (*Song et al*, 2021).

Table 6

Sexual dimorphism in parameters of phagocytic function of blood neutrophils and monocytes in intact rats

Sex	Males (10)	Females (10)	Males (10)	Females (10)	Student's statistics	
Variables	Raw values		Z-scores		Cv	t
Phagocytosis index of neutrophils, %	82,3 ± 0,7	70,1 ± 1,0	0,89 ± 0,10	-0,89 ± 0,15	0,090	9,75 ^d
Phagocytosis index of monocytes, %	8,2 ± 1,0	2,5 ± 0,2	0,78 ± 0,27	-0,77 ± 0,05	0,694	5,56 ^d
Bactericidal capacity monoc., 10 ⁶ B/L	85 ± 14	57 ± 10	0,35 ± 0,35	-0,35 ± 0,24	0,571	1,63
Killing index of neutrophils, %	54,0 ± 2,0	52,0 ± 1,7	0,17 ± 0,34	-0,17 ± 0,30	0,109	0,76
BC capacity neutrophils, 10 ⁹ Bacter/L	11,2 ± 1,1	12,7 ± 2,4	-0,13 ± 0,20	0,13 ± 0,41	0,482	0,57
Microbial count neutrophils, Bact/Ph	8,1 ± 0,1	8,5 ± 0,5	-0,15 ± 0,06	0,15 ± 0,45	0,145	0,66
Leukocytes in total, 10 ⁹ /L	9,7 ± 0,50	13,2 ± 1,7	-0,40 ± 0,12	0,40 ± 0,40	0,378	1,92
Microbial count monocytes, Bac/Phag	2,8 ± 0,1	4,0 ± 0,4	-0,58 ± 0,09	0,58 ± 0,36	0,318	3,13 ^c

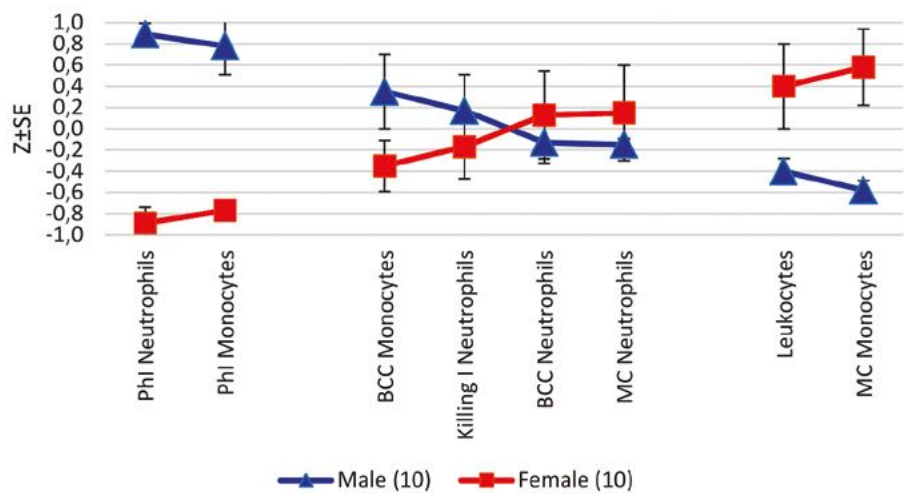


Fig. 6. Profile of Phagocytosis variables in intact rats

Another study (Fihura *et al*, 2024; Korda *et al*, 2025) also revealed a higher activity of phagocytosis of microphages in males, but a lower bactericidal capacity of monocytes in the absence of significant differences in other variables. The presented data are collected and visualized in Fig. 7. It was established that males have significantly higher levels of 11 variables: serum testosterone and triiodothyronine, percentage of epitheliocytes, endotheliocytes, and macrophages in the thymocytoqram and its entropy, percentage of lymphocytes and lymphoblasts in the splenocytoqram, phagocytosis activity of blood microphages and macrophages, as well as percentage of 0-Lymphocytes. On the other hand, females have significantly higher levels of 19 variables: serum corticosterone, catecholamines, PTH, calcitonin, aldosterone, the mass of the adrenal glands and the thickness of their fascicular, reticular and glomerular zones, the percentage of lymphocytes and lymphoblasts in the thymocytoqram, fibroblasts in the splenocytoqram and its entropy, natural killers and B-Lymphocytes in the immunocytoqram of blood and its entropy, intensity of phagocytosis of macrophages as well as reaction of blast transformation of T-Lymphocytes to phytohemagglutinin.

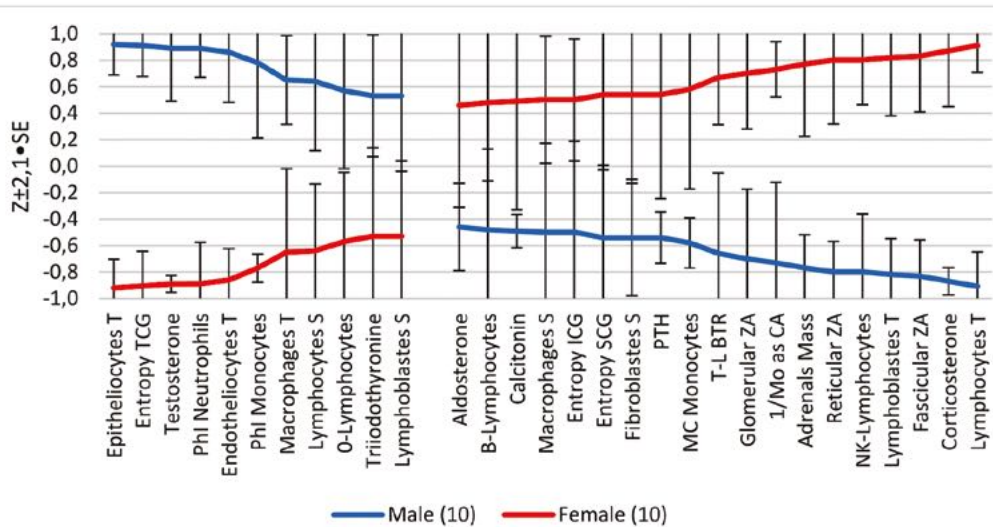


Fig. 7. Sexual dimorphism of the neuroendocrine-immune complex in intact rats

Discriminant Analysis. To identify the minimal set of variables that best discriminates between sexes, stepwise discriminant analysis (*Klecka*, 1980) was performed on the full set of measured variables. The 14 variables selected as specific endocrine-immune markers of sex (Tables 7 and 8).

Table 7

Discriminant Function Analysis Summary for Variables currently in the model
Step 14, N of vars in model: 14; Grouping: 2 groups; Wilks' Λ : 0,0014;
approx. $F(14,5) = 260$; $p < 10^{-6}$

Variables	Sex (n) and Z±SE		Parameters of Wilks' Statistics				
	Male (10)	Female (10)	Wilks Λ	Partial Λ	F-re- move (1,5)	p-le- vel	Tole- rancy
Aldosterone	−0,46 ± 0,16	0,46 ± 0,37	0,002	0,556	3,998	0,102	0,157
Reticular zone of adrenal glands	−0,80 ± 0,11	0,80 ± 0,23	0,002	0,819	1,103	0,342	0,452
Fascicular zone of adrenal glands	−0,83 ± 0,13	0,83 ± 0,20	0,013	0,101	44,36	0,001	0,058
Calcitonin	−0,49 ± 0,06	0,49 ± 0,39	0,004	0,343	9,585	0,027	0,129
Lymphocytes of Thymus	−0,91 ± 0,13	0,91 ± 0,10	0,002	0,752	1,652	0,255	0,412
Lymphoblastes of Thymus	−0,82 ± 0,13	0,82 ± 0,21	0,001	0,918	0,445	0,534	0,438
Epitheliocytes of Thymus	0,92 ± 0,11	−0,92 ± 0,10	0,002	0,654	2,646	0,165	0,265
Thymocytoqram Entropy	0,91 ± 0,11	−0,91 ± 0,13	0,006	0,235	16,26	0,010	0,152
Fibroblastes of Spleen	−0,54 ± 0,21	0,54 ± 0,32	0,003	0,487	5,277	0,070	0,095
Phagocytosis index of neutrophils	0,89 ± 0,10	−0,89 ± 0,15	0,003	0,442	6,315	0,054	0,164
Phagocytosis index of monocytes	0,78 ± 0,27	−0,77 ± 0,05	0,005	0,263	14,04	0,013	0,192
Microbial count monocytes	−0,58 ± 0,09	0,58 ± 0,36	0,002	0,712	2,019	0,215	0,078
Immunocytoqram Entropy	−0,50 ± 0,33	0,50 ± 0,22	0,004	0,391	7,794	0,038	0,052
0-Lymphocytes	0,57 ± 0,28	−0,57 ± 0,25	0,002	0,669	2,471	0,177	0,112

Table 8

Summary of stepwise analysis of discriminant variables ranked by criterion Λ

Variables currently in the model	F to enter	p-level	Λ	F-value	p-value
Epitheliocytes of Thymus	143	10-6	0,1115	143	10^{-6}
Fascicular zone of adrenal glands	28,39	10-4	0,0418	195	10^{-6}
Thymocytoqram Entropy	13,75	0,002	0,0225	232	10^{-6}
Phagocytosis index of monocytes	6,515	0,022	0,0157	236	10^{-6}
Phagocytosis index of neutrophils	5,182	0,039	0,0114	242	10^{-6}
Lymphoblastes of Thymus	6,094	0,028	0,0078	276	10^{-6}
Calcitonin	5,510	0,037	0,0053	320	10^{-6}
Immunocytoqram Entropy	2,518	0,141	0,0043	316	10^{-6}
Lymphocytes of Thymus	2,410	0,152	0,0035	317	10^{-6}

Fibroblastes of Spleen	1,253	0,292	0,0031	292	10 ⁻⁶
Aldosterone	1,019	0,342	0,0027	266	10 ⁻⁶
Microbial count monocytes	1,683	0,236	0,0022	265	10 ⁻⁶
0-Lymphocytes	1,861	0,221	0,0017	275	10 ⁻⁶
Reticular zone of adrenal glands	1,103	0,342	0,0014	260	10 ⁻⁶

Other variables, despite significant gender differences, were outside the discriminant model (Table 9), due to their duplication of discriminant information.

Table 9

Discriminant Function Analysis Summary for Variables currently not in the model

Variables	Sex (n) and Means ± SE		Parameters of Wilks' Statistics				
	Male (10)	Female (10)	Wilks Λ	Partial Λ	F-re- move (1,5)	p-le- vel	Tole- rancy
Testosterone	0,89 ± 0,19	-0,89 ± 0,03	0,001	0,993	0,028	0,877	0,213
Corticosterone	-0,87 ± 0,05	0,87 ± 0,20	0,001	0,802	0,988	0,376	0,279
Mode-1 as catecholamines	-0,73 ± 0,29	0,73 ± 0,10	0,001	0,977	0,099	0,768	0,334
Adrenals Mass	-0,77 ± 0,12	0,77 ± 0,26	0,001	0,822	0,978	0,366	0,270
Glomerular zone of adrenal glands	-0,70 ± 0,25	0,70 ± 0,20	0,001	0,969	0,128	0,739	0,464
Parathyroid hormone	-0,54 ± 0,09	0,54 ± 0,37	0,001	0,805	0,980	0,370	0,275
Triiodothyronine	0,53 ± 0,22	-0,53 ± 0,32	0,001	0,810	0,986	0,377	0,289
Macrophages of Thymus	0,65 ± 0,16	-0,65 ± 0,30	0,001	0,987	0,053	0,829	0,080
Endotheliocytes of Thymus	0,86 ± 0,36	-0,86 ± 0,23	0,001	0,820	0,984	0,376	0,249
Lymphocytes of Spleen	0,64 ± 0,25	-0,64 ± 0,24	0,001	0,997	0,011	0,920	0,077
Lymphoblastes of Spleen	0,53 ± 0,57	-0,53 ± 0,57	0,001	0,844	0,928	0,379	0,299
Macrophages of Spleen	-0,50 ± 0,32	0,50 ± 0,23	0,001	0,980	0,084	0,787	0,295
Splenocytogram Entropy	-0,54 ± 0,26	0,54 ± 0,27	0,001	0,999	0,002	0,963	0,244
Blast Transformation Reaction of T-Lymph.	-0,66 ± 0,29	0,67 ± 0,17	0,001	0,893	0,478	0,528	0,150
NK Lymphocytes of Blood	-0,80 ± 0,21	0,80 ± 0,16	0,001	0,919	0,352	0,585	0,024
B-Lymphocytes	-0,48 ± 0,29	0,48 ± 0,28	0,001	0,888	0,506	0,516	0,149

Calculation of the individual values of the discriminant root by summing the constant and products of the individual values of the discriminant variables on their Raw coefficients (Table 10) enables the visualization of each animal (Fig. 8).

Table 10

Coefficients and constant for discriminant variables

Variables currently in the model	Coefficients		
	Standar-dized	Structural	Raw
Epitheliocytes of Thymus	1,143	0,105	0,542
Thymocytogram Entropy	2,246	0,094	78,81

Phagocytosis index of neutrophils	1,847	0,085	0,660
Phagocytosis index of monocytes	1,962	0,049	0,854
0-Lymphocytes	-1,723	0,027	-0,383
Lymphocytes of Thymus	-0,777	-0,097	-0,270
Fascicular zone of adrenal glands	-3,934	-0,061	-0,081
Lymphoblastes of Thymus	-0,432	-0,058	-0,615
Reticular zone of adrenal glands	0,633	-0,054	0,068
Microbial count monocytes	-1,927	-0,027	-2,167
Fibroblastes of Spleen	2,321	-0,024	1,321
Calcitonin	-2,263	-0,022	-0,170
Immunocytoqram Entropy	-3,434	-0,022	-140,6
Aldosterone	-1,682	-0,020	-0,009
Eigenvalue	728	Constant	82,05
Canonical R = 0,9993; Wilks' Λ = 0,0014; $\chi^2_{(14)} = 72,5$; $p < 10^{-6}$			
Squared Mahalanobis Distance = 2620; $F_{(14,5)} = 260$; $p < 10^{-5}$			

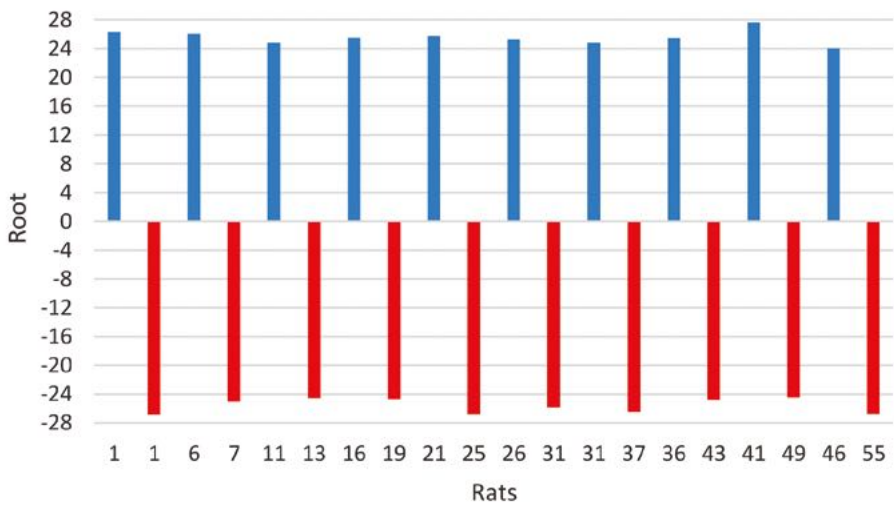


Fig. 8. Individual discriminant Roots of male and female intact rats (animal numbers are indicated)

Thus, intact rats show pronounced sexual dimorphism in a number of indicators of the endocrine and immune systems, which can be assessed quantitatively by the Mahalanobis distance ($D^2 = 2620$) and the difference between the discriminant roots (51,2). This approach will make it possible to quantitatively assess the influence of stressors and adaptogens on sexual endocrine-immune dimorphism in subsequent studies.

Another consequence of discriminant analysis is the possibility of retrospectively recognizing the sex of an animal by calculating individual classification functions. These functions are special linear combinations that maximize differences between groups and minimize dispersion within groups. An object belongs to a group with the maximum value of a function calculated by summing the constant and products of the individual values of the discriminant variables on their Classification coefficients (Table 11). Classification accuracy is 100 %.

Table 10

Coefficients and constant for discriminant variables

Sex	Male	Female
Variables	P = 0,50	P = 0,50
Epitheliocytes of Thymus	37,48	9,746
Fascicular zone of adrenal glands	5,965	10,12
Thymocytoqram Entropy	−5322	−9356
Phagocytosis index of monocytes	−58,60	−102,3
Phagocytosis index of neutrophils	−20,17	−53,95
Lymphoblastes of Thymus	−94,40	−62,93
Calcitonin	17,57	26,26
Immunocytoqram Entropy	18853	26049
Lymphocytes of Thymus	42,63	56,46
Fibroblastes of Spleen	−182,7	−250,3
Aldosterone	1,015	1,487
Microbial count monocytes	401,1	512,1
0-Lymphocytes	58,35	77,96
Reticular zone of adrenal glands	−1,016	−4,508
Constants	−8746	−12946

Conclusion. Intact Wistar rats demonstrate pronounced sexual dimorphism across a broad range of endocrine and immune parameters. Females are characterised by higher adrenocortical and adrenomedullary activity, higher calcitonin and PTH levels, greater thymic lymphocyte content, higher splenic and blood immunocytoqram entropy, enhanced macrophage phagocytic intensity, and stronger T-lymphocyte proliferative response to PHA. Males are characterised by higher testosterone and triiodothyronine levels, greater diversity of the thymic stromal microenvironment (higher thymocytoqram entropy), higher splenic lymphocyte content, higher phagocytic activity of both neutrophils and monocytes, and higher 0-lymphocyte percentage. The magnitude of sexual dimorphism can be assessed quantitatively by the Mahalanobis distance ($D^2 = 2620$), which indicates near-complete inter-group separation, and by the difference between discriminant roots (51.2), reflecting the proportion of variance attributable to sex. This quantitative framework will enable assessment of the influence of stressors and adaptogens on sexual endocrine-immune dimorphism in subsequent studies.

Using stepwise discriminant analysis, 14 variables were identified as the most informative sex-specific endocrine-immune markers, encompassing hormonal, morphological, and immunological parameters. The inclusion of both endocrine and immune variables in the discriminant function confirms that sexual dimorphism in the neuro-endocrine-immune complex is a systemic, multi-level phenomenon that cannot be adequately characterised by any single parameter or system in isolation.

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