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Quantitative assessment of the contributions of inorganic and organic components of Hertsa drinking mineral water in its effect on the neuro-endocrine-immune complex and metabolism in female rats

Walery Zukow[1], https://orcid.org/0000-0002-7675-6117; w.zukow@wp.pl
Ivan V. Savytskyi[2], https://orcid.org/0000-0002-5841-9993; prof_s.i.v@ukr.net
Dariya V. Popovych[3], https://orcid.org/0000-0002-5142-2057; darakoz@yahoo.com
Halyna Y. Kovalchuk[4], https://orcid.org/0000-0002-5261-8422; galynakovalchuk5@gmail.com
Ruslan B. Ponomarenko[5], https://orcid.org/0009-0000-4792-5949; karapuzija@gmail.com;

[1]Nicolaus Copernicus University, Torun, POLAND
[2]International Academy of Ecology and Medicine, Kyïv, UKRAINE
[3]IY Horbachevskyi National Medical University, Ternopil', UKRAINE
[4]Ivan Franko State Pedagogical University, Drohobych, UKRAINE
[5]Truskavetsian Center for Primary Medical and Sanitary Assistance of the Truskavets' City Council, Truskavets', UKRAINE

Summary

Background and aim. Our attention was drawn to the drinking mineral water from the Hertsa field (Chernivtsi oblast', Ukraine) due to its unique combination of salts, trace elements and organic substances. The aim of this study was to quantitatively assess the contributions of inorganic and organic components of this water in its effect on the neuro-endocrine-immune complex and metabolism in female rats.

Material and methods. The experiment carried out at 40 female rats Wistar line. 10 animals remained intact with free access to tap daily water, 10 formed a control group, whose members loaded with same daily water (3 mL/200g) once for 6 days, while 10 rats of the next group loaded with native water from the Hertsa field (HN) or its artificial saline analogue (HASA). The day after the completion of the treating course in all rats assessed the state of neuro-endocrine-immune complex and metabolism.

Results. The registered variables were screened to identify those in which at least two groups differed significantly. As a result of discriminant analysis, only 12 variables were included in the model, while the remaining 6 were left out of the model as carriers of duplicate/redundant discriminant information. It was found that the procedure of loading animals with plain water is not physiologically neutral, but is accompanied by changes in a number of variables as a manifestation of chronic aversive stress (increased levels of testosterone, aldosterone, Na/K serum ratio, glycemia, catalase activity in combination with decreased levels of PTH, calcium,

Ca/P serum ratio, potassium, chloriduria, natriuria, osmolarity urine, NK lymphocytes as well as the thickness of the medullary zone of adrenal glands. Taking into account this fact, it has been stated that both inorganic (salts & trace elements) and organic substances of HN and HASA reduce, eliminate or even reverse stressor effects as well as initiate a number of others, in particular a significant increase in glomerular filtration rate.
***Key words*:** Hertsa mineral water, trace elements, organic substances, neuro-endocrine-immune complex, metabolism, female rats.

Introduction

Despite the fact that it has been known since at least 1975 that over 300 studied drinking mineral waters of various mineralization (2-30 g/L) and salt (salty, alkaline, bitter, etc.) composition contain trace elements and various organic substances (5-40 mg/L) of oil or humic nature, it is still salts that are considered to be the carriers of the physiological and therapeutic activity of these waters, especially those of high and medium mineralization. Some attention is paid to trace elements, while the contribution of organic substances is ignored due to the ratio of their gross content (g vs. mg). However, due to a fortunate coincidence of circumstances - the presence of high physiological and therapeutic activity of drinking waters, which formally cannot even be considered mineral (M 0.3-0.8 g/L), but contain organic substances in their composition, the latter have become the object of research. The gold standard of therapeutic waters of this type is undoubtedly considered to be Naftussya bioactive water of the Truskavets' Spa (Ukraine), thanks to many years of experimental and clinical research of Truskavetsian Scientific School of Balneology and Phytotherapy [1-5].

Our attention was drawn to the drinking mineral water from the Hertsa field (Chernivtsi oblast', Ukraine) due to its unique combination of salts, trace elements and organic substances [1]. The aim of this study was to quantitatively assess the contributions of inorganic and organic components of this water in its effect on the neuro-endocrine-immune complex and metabolism in female rats.

Material and methods.

Participants

The experiment carried out at 40 female rats Wistar line. Weight Mean=260 g; SD=21 g.

Ethics approval

All animals were kept in room having temperature 22±2ºC, and relative humidity of 44-55% under 12/12 hour 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 (Kyïv, 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.

Study design and procedure

The study was conducted according to the algorithm of the Truskavetsian Scientific School of Balneology and Phytotherapy [1,5]. In the experiment, 10 animals remained intact with free access to tap daily water, 10 formed a control group, whose members loaded intragastrically through a tube with same daily water (3 mL/200g) once for 6 days, while 10 rats of the next group loaded with native water from the Hertsa field (Chernivtsi oblast', Ukraine) or its artificial saline analogue (without trace elements and organic substances).

The chemical composition of the applied waters (according to Truskavetsian Hydrogeological Regime-operational Station data) is given in Table 1.

Table 1. The chemical composition of the applied mineral waters

	Daily Water	Hertsa native	Hertsa Salt analogue
Electrolytes, mM/L			
Na^+	0,5	196,7	196,7
Cl^-	3,4	205	205
HCO_3^-	2,9	5,6	5,6
Ca^{2+}	3,4	3,40	3,40
Mg^{2+}	0,5	3,44	3,44
K^+	0,4	0,4	0,4
SO_4^{2-}	1,2	0,1	0,1
Trace elements, mg/L			
H_2SiO_3	5	9,88	0
H_3BO_3	0,25	42,76	0
Br	8,3	21,17	0
J	0,025	6,62	0
F	0,95	0,57	0
Organic substances, mg/L			
C org	5,0	34	0
N org	0,02	0,14	0

The day after the completion of the treating course animals were placed in individual chambers with perforated bottom for collecting daily urine.

Then in all rats, at first, assessed the state of autonomous regulation. For this purpose, under an easy ether anesthesia, for 15-20 sec ECG was recorded in the lead II, inserting needle electrodes under the skin of the legs, followed by the calculation of the parameters of the HRV: mode (Mo), amplitude of the mode (AMo) and variation scope (MxDMn) as markers of the humoral channel of regulation, sympathetic and vagal tones respectively [6].

The experiment was completed by decapitation of rats under ether anesthesia in order to collect as much blood as possible as well as removal adrenal glands, thymus and spleen.

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). An additional adaptation parameter was the old good mass index of the adrenal glands and the thickness of the glomerular, fascicular, reticular, and medullary layers, measured under a microscope on smears.

Among the metabolism parameters determined the levels in serum electrolytes: calcium (by reaction with arsenase III), magnesium (by reaction with colgamite), phosphates (phosphate-molybdate method), chloride (mercury-rhodanidine method), sodium and potassium (by flamming photometry); other metabolites: urea (urease method by reaction with phenolhypochlorite), uric acid (uricase method), middle mass molecules/medium molecular polypeptides (by spectrophotometric method), bilirubin (by diazoreaction using the Jedrashik-Kleghorn-Grof method), cholesterol (by a direct method after the classic reaction by Zlatkis-Zack), amylase (Karavay's amyloclastic method with starch substrate), glucose (glucose-oxidase method) [7]; lipid peroxidation products: diene conjugates (spectrophotometry of the heptane phase of the lipids extract [8]) and malondyaldehide (in the test with thiobarbituric acid

[9]), antioxidant enzymes: superoxide dismutase erythrocytes (according to the degree of inhibition of reduction of nitroblue tetrazolium in the presence of N-methylphenazonium metasulphate and NADH [10]) and catalase plasma (at the rate of decomposition of hydrogen peroxide [11]). We calculated antioxidant activity (AOA) by formula: $AOA=[(SOD•Cat)/(DC•MDA)]^{0,25}$.

Most of the listed parameters of metabolism were also determined in daily urine followed by calculating its osmolarity.

The metabolic analyzes were carried out according to the instructions. The analyzers "Pointe-180" ("Scientific", USA) and "Reflotron" (Boehringer Mannheim, BRD) were used with appropriate sets and a flaming spectrophotometer "СФ-47".

The parameters of immunity were determined, as described in the manual [12]. First of all, calculated of Leukocytogram (LCG), ie the percentage of lymphocytes (L), monocytes (M), eosinophils (E), basophils (B), rod-shaped (RN) and polymorphonucleary (PMNN) neutrophils. Based on these data, the Entropy of the Leukocytogram (hLCG) was calculated according to the equation derived by Popovych IL [13,14,15] on the basis of the classical Shannon's CE [16] equation:

$hLCG = - (L•\log_2 L+M•\log_2 M+E•\log_2 E+B•\log_2 B+RN•\log_2 RN+PMNN•\log_2 PMNN)/\log_2 6$.

The percentage of theophylline-resistant (TR) and theophylline-susceptible (TS) T-lymphocytes, B-lymphocytes, plasma cells (Pla), and natural killers (NK) were identified. The percentage of 0-lymphocytes calculated by balance method. For these components the Entropy of the Immunocytogram (hICG) was calculated by Popovych IL [13,14,15] equation:

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

About the condition of the phagocytic function of neutrophils (microphages) and monocytes (macrophages) were judged by the phagocytosis index (percentage of cells, in which found microbes), the microbial count (number of microbes absorbed by one phagocyte) and the killing index (percentage of dead microbes) for *Staphylococcus aureus* (ATCC N25423 F49). Based on these parameters, taking into account the absolute content of neutrophils and monocytes, their bactericidal capacity was calculated (BCC N&M) [17].

The Spleen and Thymus were weighed and made smears-imprints for counting Thymocytogram and Splenocytogram [17]. The components of the Thymocytogram (TCG) are lymphocytes (Lc), lymphoblastes (Lb), reticulocytes (Ret), macrophages (Mac), basophiles (B), endotheliocytes (En), epitheliocytes (Ep), and Hassal's corpuscles (H). The Splenocytogram (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 [13,14,15]:

$hTCG = -(Lc•\log_2 Lc+Lb•\log_2 Lb+Ret•\log_2 Ret+Mac•\log_2 Mac+B•\log_2 B+En•\log_2 En+Ep•\log_2 Ep+H•\log_2 H)/\log_2 8$;

$hSCG = -(Lc•\log_2 Lc+Lb•\log_2 Lb+Pla•\log_2 Pla+R•\log_2 R+Ma•\log_2 Ma+F•\log_2 F+Mi•\log_2 Mi+Eo•\log_2 Eo)/\log_2 8$.

Statistical analysis

Statistical processing was performed using a software package "Microsoft Excell" and "Statistica 6.4 StatSoft Inc" (Tulsa, OK, USA).

Results

Adhering to the Truskavetsian Scientific School's analytical algorithm [1,5,18], the actual/raw variables were normalized by recalculation by the equation:

$Z = (V – N)/SD$, where

V is the actual value; N is the normal (at intact rats) value; SD is standard deviation at intact rats. Z-score normalization enabling comparison of diverse variables on a uniform scale.

Next, the registered variables were screened to identify those in which at least two groups differed significantly, followed by the construction of their profiles (Fig. 1).

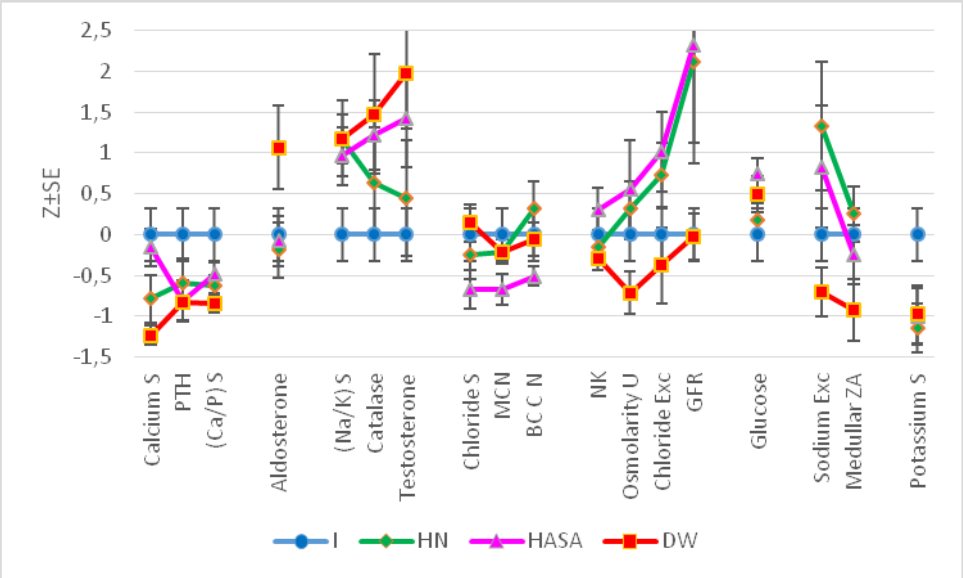


Fig. 1. Z-score profiles of the variables of intact animals (I) and those loaded with daily water (DW), native Hertsa water (HN) and its artificial saline analogue (HASA). See also Table 5.

At the next stage, the selected variables were subjected to discriminant analysis (forward stepwise method [19]) (Tables 2 and 3). As a result, only 12 variables were included in the discriminant model, while the remaining 6 were left out of the model as carriers of duplicate/redundant discriminant information.

Table 2. Discriminant Function Analysis Summary for Variables, their actual levels and Z-scores (Mean±SE)

Step 12, N of vars in model: 12; Grouping: 4 grs; Wilks' Λ: 0,0926; approx. $F_{(36,7)}$=2,6; $p<10^{-3}$

Variables currently in the model	Groups (n)				Parameters of Wilk's Statistics				
	Intact (10)	Hertsa native (10)	Hertsa ASA (10)	Daily water (10)	Wilks Λ	Partial Λ	F-remove (3,25)	p-level	Tolerancy
Aldosterone serum, pM/L	813 149	722 161	776 146	1315 241	0,112	0,826	1,75	0,182	0,323
Testosterone Serum, nM/L	3,93 0,34	4,41 0,76	5,44 0,63	6,04 0,72	0,119	0,776	2,41	0,091	0,484
Parathyroid hormone, µg/L	200 21	161 20	146 16	145 16	0,129	0,715	3,33	0,036	0,337
Medullary Zone of Adrenal glands, µM	94 10	102 10	87 11	65 12	0,112	0,828 4122	1,726 071	0,187 2623	0,375 609
$(Ca/P)^{0,5}$ Serum ratio	2,56 0,37	1,83 0,15	1,99 0,18	1,58 0,15	0,119	0,775	2,42	0,090	0,265
$(Na/K)^{0,5}$ Serum ratio	5,57 0,17	6,21 0,17	6,10 0,20	6,2 0,25	0,113	0,822	1,81	0,171	0,051
Calcium Serum, mM/L	3,35 0,32	2,54 0,30	3,19 0,23	2,08 0,12	0,123	0,750	2,78	0,062	0,389
Potassium Serum, mM/L	4,23 0,22	3,42 0,21	3,52 0,25	3,54 0,25	0,113	0,822	1,81	0,172	0,050

Sodium Excretion, µM/24h•100g	135 27	246 66	205 63	76 25	0,103	0,903	0,899	0,456	0,308
Glucose Serum, mM/L	4,95 0,35	5,15 0,22	5,77 0,20	5,49 0,24	0,145	0,640	4,68	0,010	0,450
Catalase Serum, µM/L•h	103 9	121 19	138 12	145 21	0,123	0,752	2,75	0,064	0,570
Microbial Count of Neutrophils, Bacteria/Phagoc	8,6 0,6	8,2 0,3	7,3 0,4	8,2 0,3	0,098	0,944	0,498	0,687	0,464
Variables currently not in the model	**Intact (10)**	**Hertsa native (10)**	**Hertsa ASA (10)**	**Daily water (10)**	Wilks Λ	Partial Λ	F to enter	p-level	Tolerancy
Chloride Serum, mM/L	94,3 2,3	92,5 2,1	89,5 1,7	95,4 1,6	0,083	0,896	0,93	0,443	0,256
Chloride Excretion, µM/24h•100g	144 31	216 39	243 48	107 45	0,092	0,999	0,01	0,998	0,305
Osmolarity Urine, mOsm/L	559 44	604 46	636 85	460 36	0,090	0,977	0,19	0,903	0,406
Glomerular Filtration, µL/min•100g	86 10	151 38	157 37	85 9	0,084	0,908	0,81	0,499	0,605
NK Lymphocytes Blood, %	15,6 0,9	15,2 0,4	16,5 0,7	14,8 0,4	0,089	0,958	0,35	0,788	0,239
Bactericide Capacity of Neutrophils, 10^9 Bacter/L	11,56 2,29	13,91 2,46	7,85 0,89	11,14 1,45	0,089	0,965	0,29	0,829	0,723

Table 3. Summary of Stepwise Analysis. Variables ranked by criterion Lambda

Variables currently in the model	F to enter	p-level	Λ	F-value	p-level
Calcium Serum, mM/L	5,270	0,004	0,695	5,270	0,004067
Parathyroid hormone, µg/L	3,129	0,038	0,548	4,095	0,001407
Microbial Count of Neutrophils, Bact/Phag	3,186	0,036	0,428	3,847	0,000420
Sodium Excretion, µM/24h•100g	2,937	0,048	0,338	3,705	0,000157
Glucose Serum, mM/L	2,517	0,076	0,273	3,551	0,000088
$(Na/K)^{0,5}$ Serum ratio	2,294	0,097	0,223	3,422	0,000060
Catalase Serum, µM/L•h	2,038	0,130	0,186	3,293	0,000049
Potassium Serum, mM/L	1,346	0,279	0,163	3,068	0,000078
$(Ca/P)^{0,5}$ Serum ratio	1,274	0,302	0,143	2,883	0,000127
Aldosterone serum, pM/L	1,241	0,314	0,126	2,732	0,000193
Testosterone Serum, nM/L	1,107	0,364	0,112	2,587	0,000327
Medullary Zone of Adrenal glands, µM	1,726	0,187	0,093	2,565	0,000301

The dividing information contained in 12 variables is condensed in 3 canonical discriminant roots. The major root contains 54,5% of discriminative opportunities (r*=0,824; Wilks' Λ=0,093; $\chi^2_{(36)}$=74; p=0,0002), the second root - 30,7% (r*=0,738; Wilks' Λ=0,289; $\chi^2_{(22)}$=38; p=0,016), and the third root - 14,8% (r*=0,604; Wilks' Λ=0,635; $\chi^2_{(10)}$=14; p=0,169).

At the next stage, using raw coefficients and constants (Table 4), individual values of discriminant roots were calculated, which allowed to visualize each rat in the information field of these roots (Fig. 5).

Table 4. Standardized and raw coefficients and constants for discriminant variables

Coefficients	**Standardized**			**Raw**		
Variables	Root 1	Root 2	Root 3	Root 1	Root 2	Root 3
Calcium Serum, mM/L	-0,871	0,368	0,383	-1,07	0,454	0,474
Parathyroid hormone, µg/L	-1,099	-0,163	-0,173	-0,019	-0,0028	-0,0030
Microbial Count of Neutrophils, Bact/Phag	-0,130	-0,218	0,480	-0,101	-0,169	0,373
Sodium Excretion, µM/24h•100g	0,058	0,682	-0,408	0,0004	0,0044	-0,0026
Glucose Serum, mM/L	0,482	1,033	0,406	0,593	1,269	0,499

$(Na/K)^{0,5}$ Serum ratio	1,369	-2,027	-0,079	2,161	-3,199	-0,125
Catalase Serum, µM/L•h	0,702	-0,262	0,413	0,014	-0,005	0,008
Potassium Serum, mM/L	0,997	-2,271	0,473	1,349	-3,074	0,640
$(Ca/P)^{0,5}$ Serum ratio	0,846	0,213	0,964	2,080	0,524	2,371
Aldosterone serum, pM/L	-0,430	-0,801	-0,417	-0,428	-0,798	-0,416
Testosterone Serum, nM/L	0,589	0,078	0,783	0,298	0,039	0,395
Medullary Zone of Adrenals gland, µM	-0,656	-0,200	-0,625	-0,020	-0,006	-0,019
		Constants		-19,00	24,95	-12,79
		Eigenvalues		2,123	1,196	0,575
	Cumulative Proportions			0,545	0,852	1

Ignoring mathematical formalities, we still included in Table 5 also 6 variables that were not included in the discriminant model, since they organically fit into the factorial structure of the roots.

As can be seen in Fig. 2, along the major root axis, the leftmost position is occupied by intact animals, and the rightmost position is occupied by rats loaded with daily water. This reflects the maximum for the sample decrease in the control rats in the levels of calciemia, PTH and Ca/P serum ratio, on the one hand, and the maximum increase in the levels of testosterone, aldosterone, Na/K serum ratio as well as catalase activity - on the other hand. The rats of the other two groups occupy intermediate positions, intermixing with each other.

However, they are separated along the axis of the second root. The top position of rats loaded with Hertsa ASA reflects the maximum reduced levels of chloridemia, the intensity of neutrophil phagocytosis and their bactericidal ability in combination with the maximum for the sample levels of natural killer cells, glycemia, chloriduria and urine osmolarity, as well as glomerular filtration.

Table 5. Factor Structure Matrix (Correlations Variables-Canonical Roots), Means of Roots and Z-scores of Variables

Variables	**Correlations Variables-Roots**			**Intact rats (10)**	**Hertsa native (10)**	**Hertsa ASA (10)**	**Daily water (10)**
Root 1 (54,5%)	**R1**	**R2**	**R3**	**-2,07**	-0,33	0,78	**1,63**
Calcium Serum	**-0,309**	0,332	0,428	**0±0,32**	-0,79±0,29	-0,16±0,23	**-1,24±0,12**
Parathyroid hormone	**-0,268**	-0,086	0,084	**0±0,32**	-0,59±0,30	-0,81±0,24	**-0,83±0,24**
$(Ca/P)^{0,5}$ Serum ratio	**-0,209**	0,160	0,345	**0±0,32**	-0,63±0,13	-0,49±0,15	**-0,84±0,12**
Aldosterone serum	**0,198**	-0,283	0,154	**0±0,32**	-0,19±0,34	-0,08±0,31	**1,07±0,51**
$(Na/K)^{0,5}$ Serum ratio	**0,258**	0,056	-0,300	**0±0,32**	1,17±0,30	0,96±0,36	**1,18±0,46**
Catalase Serum	**0,231**	0,013	0,032	**0±0,32**	0,64±0,67	1,22±0,42	**1,48±0,74**
Testosterone Serum	**0,291**	-0,048	0,129	**0±0,32**	0,45±0,71	1,42±0,59	**1,97±0,67**
Root 2 (30,7%)	**R1**	**R2**	**R3**	-0,51	0,32	**1,49**	-1,30
Chloride Serum				0±0,32	-0,25±0,29	**-0,67±0,24**	0,14±0,23
Microbial Count of Neutr	-0,155	**-0,280**	-0,112	0±0,32	-0,21±0,15	**-0,68±0,19**	-0,21±0,15
Bactericide Capacity Neut				0±0,32	0,32±0,34	**-0,51±0,12**	-0,06±0,20
NK Lymphocytes Blood				0±0,32	-0,16±0,15	**0,31±0,26**	-0,30±0,14
Osmolarity Urine				0±0,32	0,32±0,33	**0,55±0,61**	-0,72±0,26
Chloride Excretion				0±0,32	0,73±0,40	**1,01±0,49**	-0,38±0,46
Glomerular Filtration				0±0,32	2,12±1,25	**2,32±1,20**	-0,03±0,28
Glucose Serum	0,233	**0,167**	0,173	0±0,32	0,18±0,20	**0,75±0,18**	0,49±0,22
Root 3 (14,8%)	**R1**	**R2**	**R3**	0,52	**-1,21**	0,56	0,14
Sodium Excretion	-0,066	0,323	**-0,323**	0±0,32	**1,33±0,79**	0,83±0,75	-0,70±0,30
Medullary Zone of AG	-0,197	0,186	**-0,231**	0±0,32	**0,25±0,34**	-0,25±0,36	-0,93±0,38
Potassium Serum	-0,249	-0,135	**0,322**	0±0,32	**-1,15±0,30**	-1,01±0,35	-0,98±0,35

Additional demarcation of these two groups occurs along the axis of the third radical, the lowest position of which in rats fed native Hertsa water reflects their maximum level of

natriuresis in combination with the minimum level of kaliemia, as well as the maximum for the sample thickness of the adrenal medullary zone.

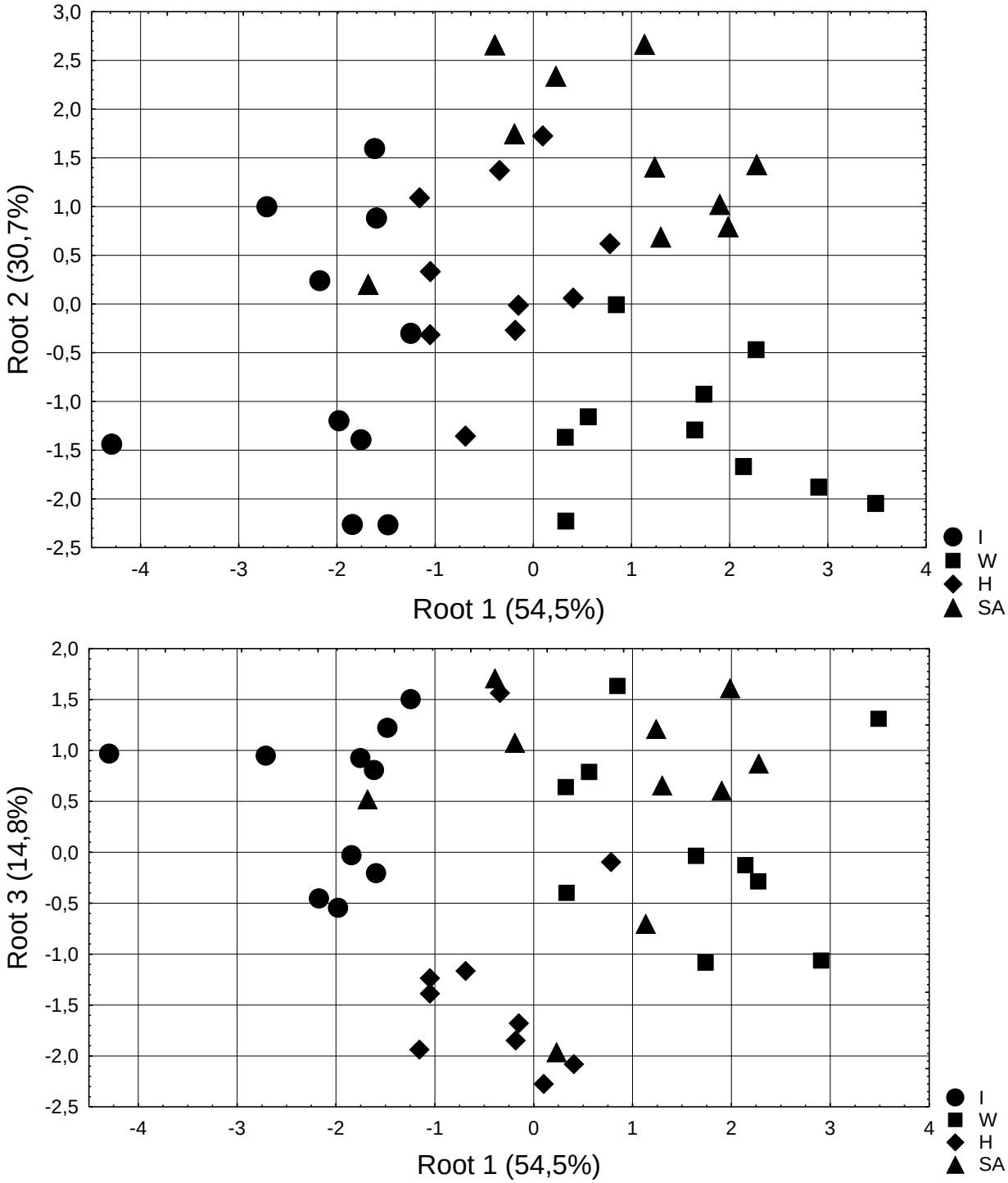


Fig. 2. Individual Roots of Intact rats (circle) and loaded by Daily Water (square), Hertsa Water (rhomb) and its Saline Analogue (triangle)

The separation of the four groups/clusters in the information field of the three roots, despite the presence of a number of mixings, is quite clear. This is documented, firstly, by calculating the Mahalanobis distances between clusters (Table 6).

Table 6. Squares of Mahalanobis distances between clusters (above the diagonal) and F-criteria (df=12,3) and p-levels (below the diagonal)

Groups	Intact rats (10)	Daily water (10)	Hertsa native (10)	Hertsa ASA (10)
Intact Rats (10)	0	14,4	6,7	12,2
Daily Water (10)	4,17 0,001	0	8,3	8,7
Hertsa Native (10)	1,94 0,079	2,41 0,031	0	5,8
Hertsa ASA (10)	3,52 0,004	2,52 0,024	1,67 0,136	0

The second evidence of a clear demarcation of clusters is the result of retrospective classification based on coefficients and constants of classification functions (Table 7).

Table 7. Coefficients and constants of classification functions

Groups	Intact rats	Daily water	Hertsa native	Hertsa ASA
Variables	p=,25	p=,25	p=,25	p=,25
Calcium Serum, mM/L	-1,053	-5,568	-3,357	-3,184
Parathyroid hormone, µg/L	-0,146	-0,214	-0,177	-0,207
Microbial Count of Neutrophils, Bact/Phag	11,36	10,98	10,39	10,74
Sodium Excretion, µM/24h•100g	0,158	0,157	0,167	0,168
Glucose Serum, mM/L	-2,289	-1,291	-1,058	1,972
$(Na/K)^{0,5}$ Serum ratio	435,2	445,8	436,5	435,0
Catalase Serum, µM/L•h	0,381	0,435	0,387	0,411
Potassium Serum, mM/L	368,4	375,6	367,0	366,1
$(Ca/P)^{0,5}$ Serum ratio	55,24	61,62	55,19	62,33
Aldosterone serum, pM/L	28,42	27,62	27,72	25,58
Testosterone Serum, nM/L	12,01	12,93	11,88	12,95
Medullar Zone of Adrenals gland, µM	-0,455	-0,517	-0,462	-0,525
Constants	-2130	-2215	-2119	-2134

The correct of classification is 87,5% (Table 8).

Table 8. Classification matrix

	Rows: Observed classifications Columns: Predicted classifications				
Group	Percent Correct	I p=,25	DW p=,25	H p=,25	HASA p=,25
I	100,0	10	0	0	0
DW	90,0	0	9	0	1
H	80,0	0	0	8	2
HASA	80,0	1	0	1	8
Total	87,5	11	9	9	11

The demarcation of clusters is visualized more clearly after calculating their centroids (Fig. 3).

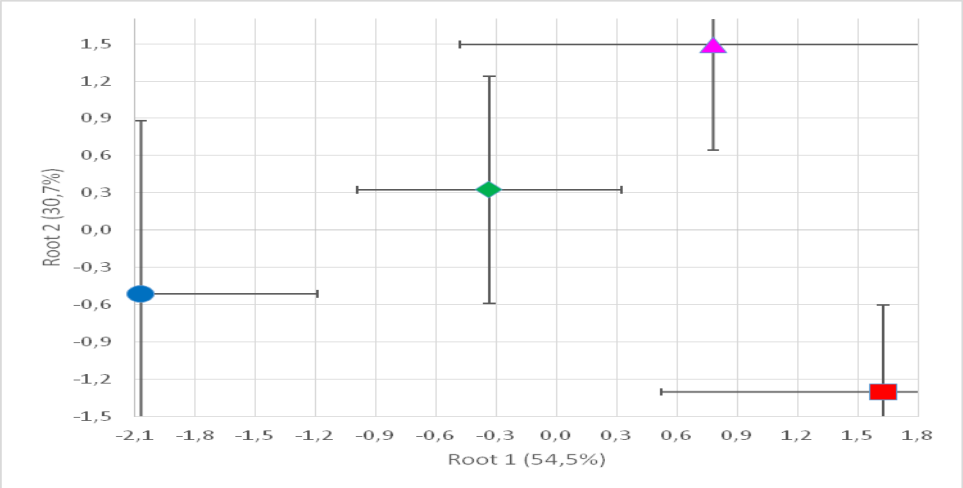


Fig. 3a. Centroids (Mean±SD) of Roots of Intact rats (circle) and loaded by Daily Water (square), Hertsa Water (rhomb) and its Saline Analogue (triangle)

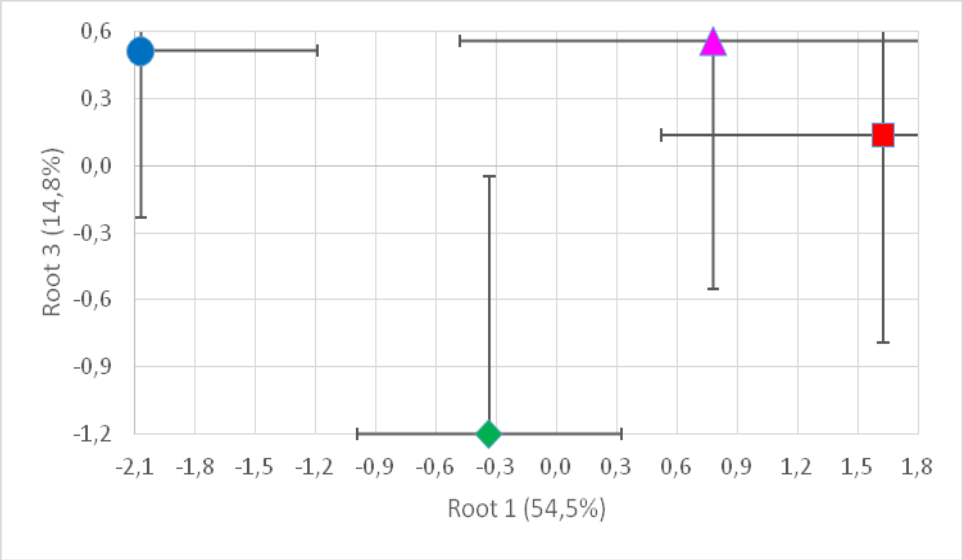


Fig. 3b. Centroids (Mean±SD) of Roots of Intact rats (circle) and loaded by Daily Water (square), Hertsa Water (rhomb) and its Saline Analogue (triangle)

Further, calculating the algebraic differences between the centroids of the experimental groups and the intact group allows us to quantitatively assess the effects of the applied factors (Fig. 4).

Since the rats in the control group were loaded with the same tap water as the intact group, the detected changes in a number of variables should be attributed to chronic aversive stress caused by fixing the animal in the experimenter's hand and inserting a tube into the esophagus for loading water [1,5].

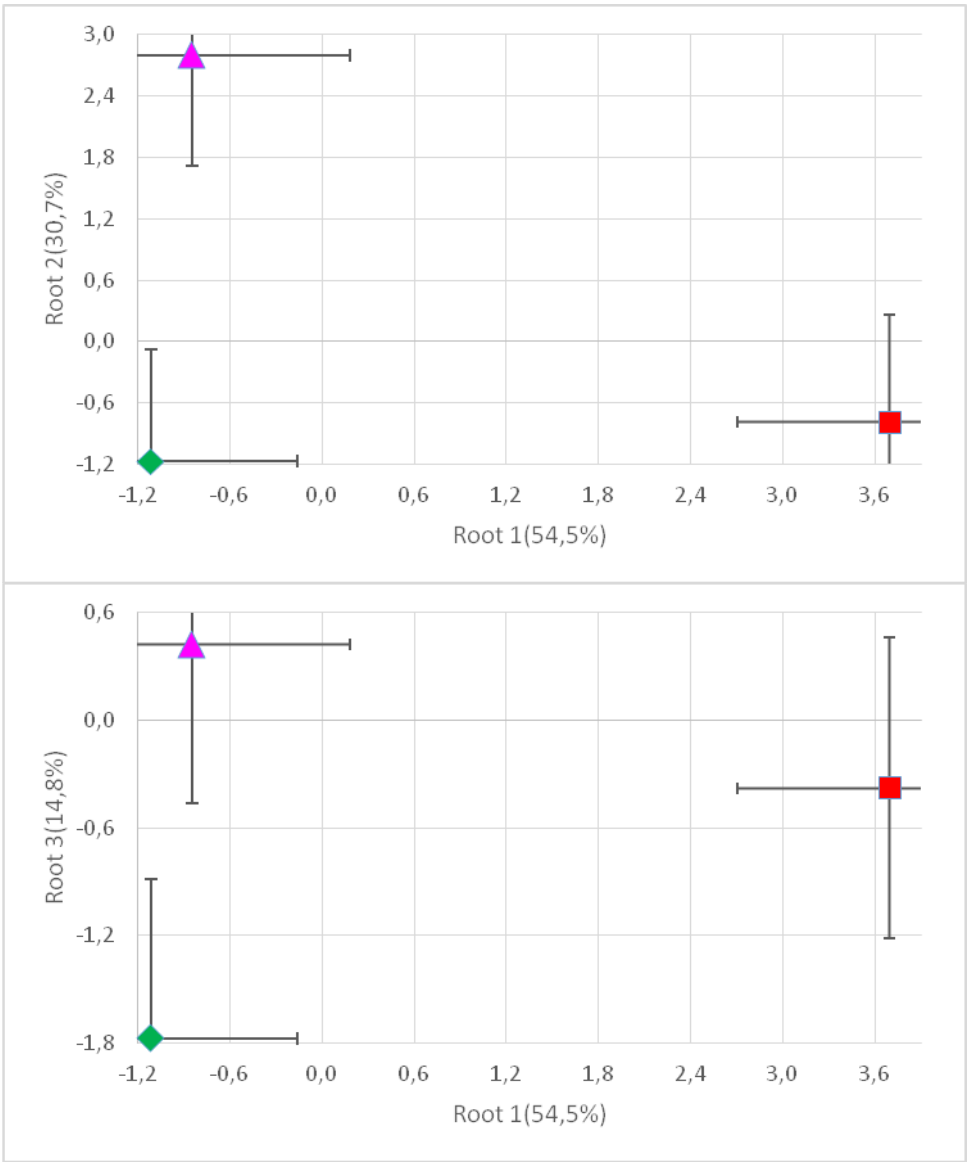


Fig. 4. Effects (Mean±SD) of Daily Water (square), Hertsa Water (rhomb) and its Salt Analogue (triangle)

The most pronounced manifestations of chronic stress in this situation are increased levels of testosterone, aldosterone, Na/K serum ratio as well as catalase activity in combination with decreased levels of PTH, calciemia, and Ca/P serum ratio, information about which is condensed in the major root (Figs. 1 and 5 as well as Table 5). The presence of salts in HASA, primarily NaCl as well as trace elements, somewhat mitigates the listed manifestations of stress, and the combination of inorganic and organic substances in the composition of native Hertsa water determines its noticeable stress-limiting effect. Interestingly, the partial stress-limiting effects of salts&trace elements and hydrocarbons are unidirectional and approximately the same.

Other, less pronounced, manifestations of stress are a decrease in urine osmolarity in general and its components of chloriduria in particular as well as NK lymphocytes level in combination with an increase in the level of glycemia, information about which is condensed in the second root. Salts&trace elements cause enhancing hyperglycemia, reversion of stressor effects on urine osmolarity and chloride excretion as well as NK level, at the same time, they initiate a decrease in the intensity of phagocytosis and bactericide capacity of neutrophils as well as chloridemia in combination with a pronounced increase in glomerular filtration rate.

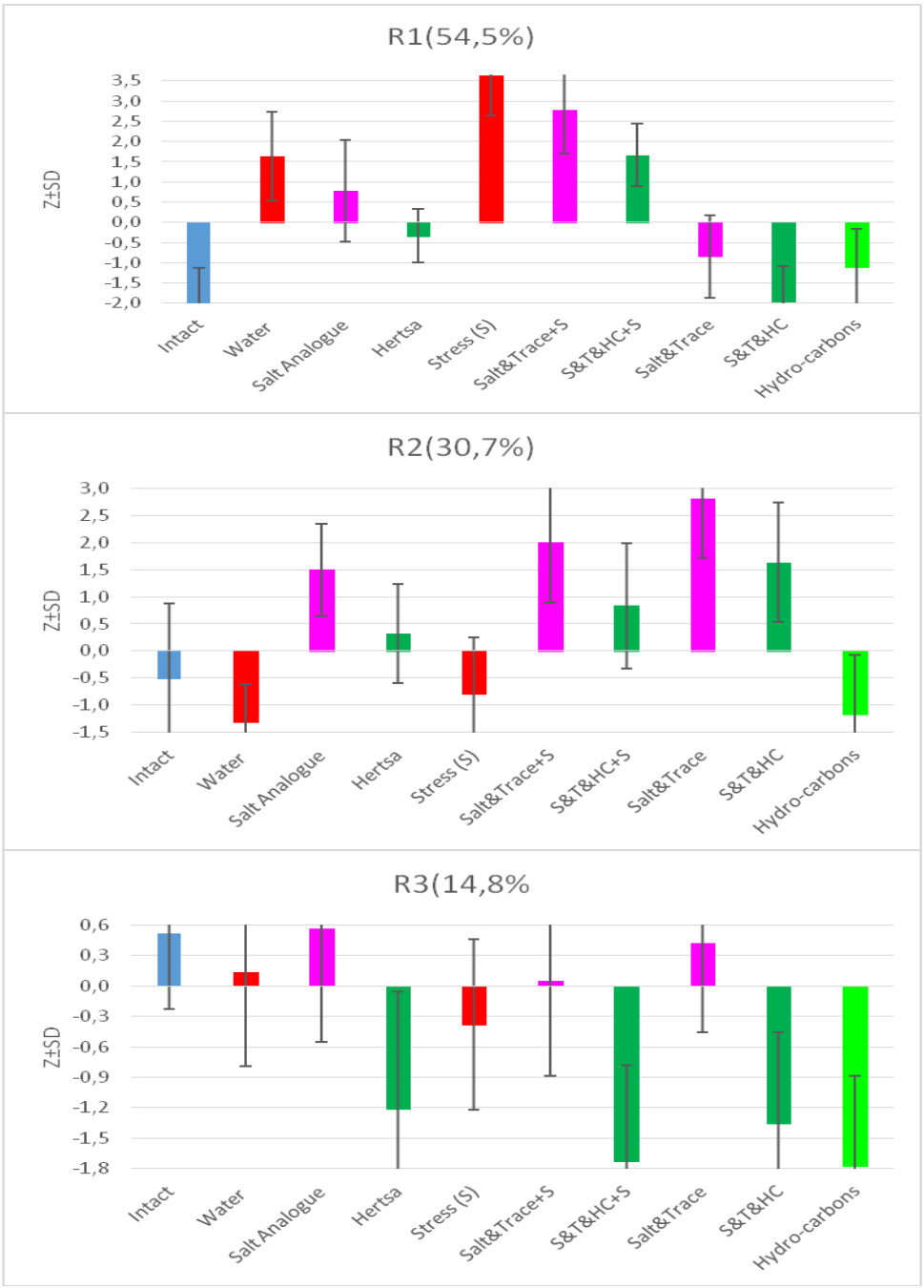


Fig. 5. Centroids (Mean±SE) of Roots of Intact rats and loaded by Daily Water, Hertsa Water, its Salt Analogue; and simulated effects of chronic stress per se, stress&salts&trace elements, stress&salts&trace elements&hydrocarbons as well as salts&trace elements per se, salts&trace elements&hydrocarbons per se and hydrocarbons per se

The combination of inorganic and organic substances in the composition of native Hertsa water to one degree or another reduces or eliminates the effects of salts & trace elements on the listed variables, with the exception of glomerular filtration, i.e. the partial effects of inorganic and organic substances in native Hertsa water are directed oppositely.

Instead, both native Hertsa water and HASA to approximately the same extent, firstly, reverse stress hyponatremia into hypernatremia, secondly, level out the decrease in the thickness of the medullary zone of adrenal glands, and thirdly, only slightly deepen hypokalemia. Thus, the partial stress-limiting effects of salts&trace elements and hydrocarbons

with respect to the variables condensed in the third root are again unidirectional and approximately the same.

It is time to analyze the obtained results in line with the concepts of the neuro-endocrine-immune complex [1,5,20] and the functional-metabolic continuum [21].

Taking hormonal variables as factorial features, and metabolic and immune variables as outcome features (Tables 9-11), we will conduct a canonical correlation analysis.

Table 9. Matrix of correlations between hormonal and Metabolic&Immune variables

Hormonal variables Metabolic&Immune variables	Aldosterone, pM/L	PTH, µg/L	Medullary ZAG, µM	Testosterone, nM/L
Sodium Excretion, µM/24h•100g	-0,665	0,073	0,628	-0,047
Osmolarity Urine, mOsm/L	-0,620	0,106	0,513	0,029
Chloride Excretion, µM/24h•100g	-0,599	0,022	0,406	-0,038
Potassium Serum, mM/L	-0,179	0,008	0,217	-0,096
Glucose Serum, mM/L	0,512	-0,201	-0,165	0,068
$(Na/K)^{0,5}$ Serum ratio	0,166	-0,009	-0,231	0,075
$(Ca/P)^{0,5}$ Serum ratio	-0,172	0,714	0,040	-0,318
Calcium Serum, mM/L	-0,179	-0,108	0,027	-0,243
Chloride Serum, mM/L	0,002	0,205	0,084	-0,010
Glomerular Filtration, µL/min•100g	-0,064	-0,155	-0,005	-0,127
Microbial Count of Neutrophils, Bacteria/Phagoc	-0,162	0,034	0,298	-0,313
Catalase Serum, µM/L•h	-0,076	-0,023	0,273	0,184
Bactericide Capacity of Neutrophils, 10^9 Bacter/L	-0,068	-0,097	0,201	0,067
NK Lymphocytes Blood, %	0,115	0,081	-0,298	0,119

Table 10. Factor structure of first pair of Hormonal and Metabolic&Immune Roots

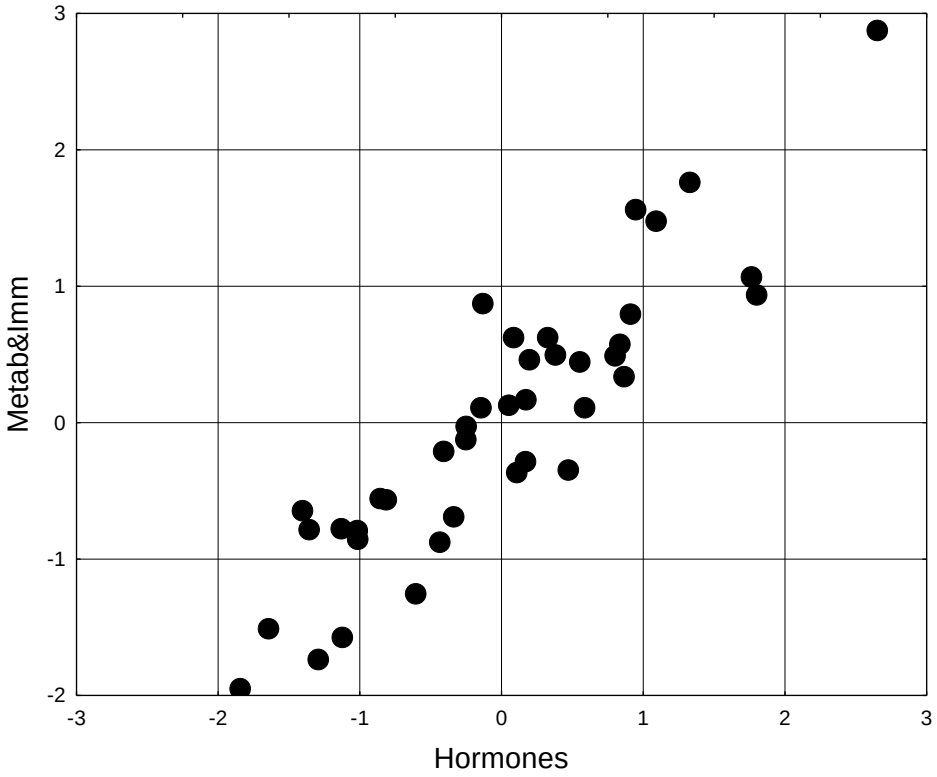
Hormones	Root 1
PTH, µg/L	0,902
Aldosterone, pM/L	0,245
Testosterone, nM/L	-0,283
Metabolic&Immune variables	Root 1
$(Ca/P)^{0,5}$ Serum ratio	0,723
Chloride Serum, mM/L	0,224
Glomerular Filtration, µL/min•100g	-0,160
Chloride Excretion, µM/24h•100g	-0,226
Sodium Excretion, µM/24h•100g	-0,175
Osmolarity Urine, mOsm/L	-0,153
Calcium Serum, mM/L	-0,139
Bactericide Capacity of Neutrophils, 10^9 Bacter/L	-0,123

Table 11. Factor structure of second pair of Hormonal and Metabolic&Immune Roots

Hormones	Root 2
Aldosterone, pM/L	0,925
Medullary ZAG, µM	-0,743
Testosterone, nM/L	0,305
PTH, µg/L	-0,299
Metabolic&Immune variables	Root 2
Sodium Excretion, µM/24h•100g	-0,807
Osmolarity Urine, mOsm/L	-0,707
Chloride Excretion, µM/24h•100g	-0,649
Potassium Serum, mM/L	-0,258
Glucose Serum, mM/L	0,514
$(Na/K)^{0,5}$ Serum ratio	0,249
Microbial Count of Neutrophils, Bacteria/Phagoc	-0,342

Bactericide Capacity of Neutrophils, 10^9 Bacter/L	**-0,106**
Catalase Serum, µM/L•h	**-0,125**
NK Lymphocytes Blood, %	**0,236**
$(Ca/P)^{0,5}$ Serum ratio	**-0,346**
Calcium Serum, mM/L	**-0,197**

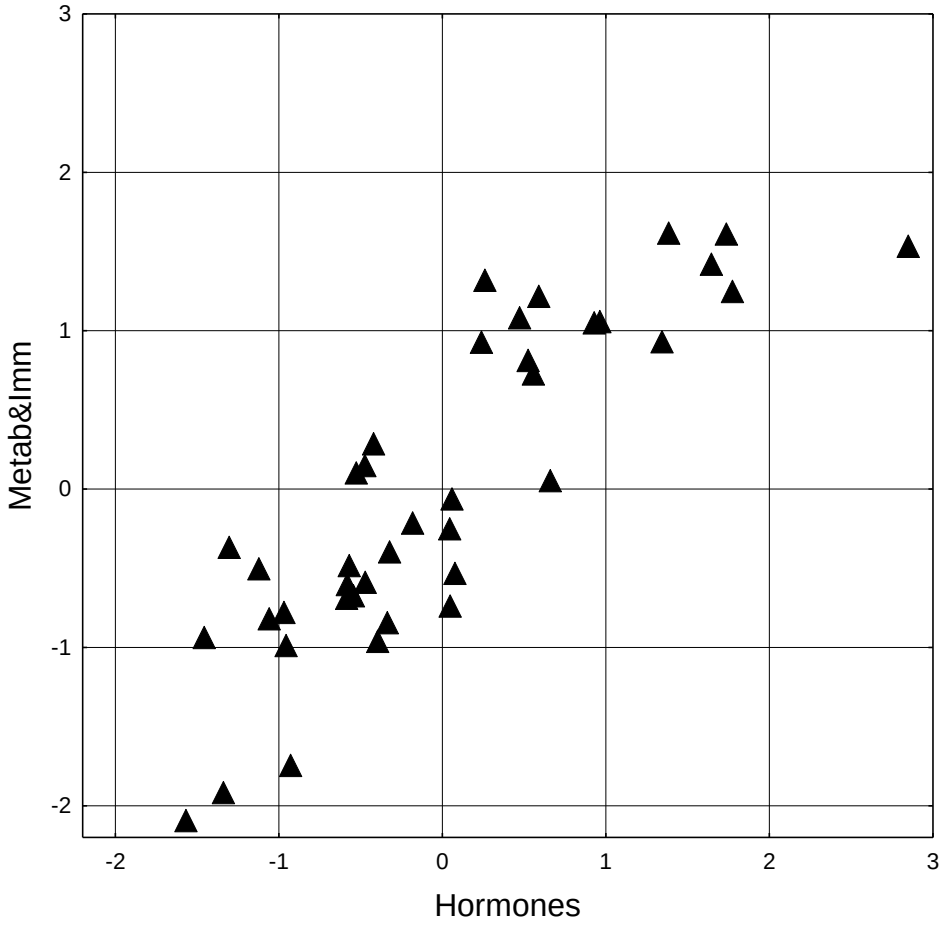
The analysis revealed two pairs of canonical roots. The hormonal root of the first pair receives the maximum factor load from PTH, and moderate loads from aldosterone and testosterone (Table 10). Taken together, these hormones determine the parameters of calcium, phosphate, sodium and chloride exchange as well as Bactericide Capacity of Neutrophils by 81.3% (Fig. 6).



R=0,902; R^2=0,813; $\chi^2_{(56)}$=121; p=10^{-6}; Λ Prime=0,0165

Fig. 6. Scatterplot of canonical correlation between Hormonal (X-line) and Metabolic&Immune (Y-line) parameters. First pair of Roots

The factor structure of the second hormonal root is represented to the maximum extent by aldosteronemia and the thickness of the adrenal medullary zone (Table 11), which is a source of circulating catecholamines, to the greatest extent by adrenaline. Together with testosterone and PTH, they determine electrolyte exchange as well as glycemia, catalase activity and immunity parameters by 74.2% (Fig. 7).



R=0,862; R^2=0,742; $\chi^2_{(39)}$=72; p=0,0011; Λ Prime=0,0880

Fig. 7. Scatterplot of canonical correlation between Hormonal (X-line) and Metabolic&Immune (Y-line) parameters. Second pair of Roots

It seems that chronic aversive stress factors cause the release of aldosterone, testosterone, and catecholamines from the glomerular, reticular (females!) and medullary layers of the adrenal glands into the blood, but in turn inhibit the release of TTH.

The intake of significant amounts of NaCl prevents stress-induced aldosterone release and significantly reduces the release of catecholamines and testosterone, without affecting PTH release. This is accompanied by corresponding changes in electrolytes exchange and immunity. The pronounced increase in GFR, caused by the intake of NaCl [22], is noteworthy.

Additional intake of organic substances prevents the release of catecholamines and reduces stress-induced changes in blood levels of PTH and testosterone, but not aldosterone.

Unfortunately, we were unable to identify individual classes of organic substances in the native Hertsa water. Therefore, we are forced to speculate that the composition of organic substances is to some extent similar to such Naftussya bioactive water and/or Ukrainian Phytocompositions [1,5,23].

Based on the structural analogue, the corresponding chemicals act through cortisol, testosterone, catecholamines, and polyunsaturated fatty acids receptors [24]. At the same time, the idea of the leading role in the implementation of the effects of phytoadaptogens of the ubiquitous aryl hydrocarbon receptor is gaining popularity and recognition [25,26,27].

Both Naftussya bioactive water [1,28,29,30] and Ukrainian Phytocompositions “Balm Kryms'kyĭ” [31], “Balm Truskavets’” [5,32-42], ATINE [23,43-45] have adaptogenic stress-limiting effects in rats and humans.

Discussion

The present study demonstrates that the procedure of loading animals with plain water is not physiologically neutral, but is accompanied by changes in numerous variables as a manifestation of chronic aversive stress. This finding aligns with previous observations by Flyunt et al. [2,4] regarding stress responses in experimental balneotherapy models. The most pronounced manifestations of chronic stress in our experiment included increased levels of testosterone, aldosterone, Na/K serum ratio, and catalase activity, combined with decreased levels of PTH, calciemia, and Ca/P serum ratio—changes that are consistent with the stress-induced activation of the hypothalamic-pituitary-adrenal axis described by Besedovsky and del Rey [20].

Stress-Limiting Effects of Hertsa Mineral Water Components

Our discriminant analysis revealed that both inorganic components (salts and trace elements) and organic substances of Hertsa native water (HN) and its artificial saline analogue (HASA) reduce, eliminate, or even reverse stressor effects. This adaptogenic activity is consistent with the well-documented properties of Naftussya bioactive water from Truskavets' Spa [1,3,5,28-30], which shares similar organic composition characteristics. The stress-limiting effects observed in our study support the concept of balneotherapy as a modulator of the neuro-endocrine-immune complex, as extensively described by Popovych et al. [1,13,14].

The partial stress-limiting effects of salts, trace elements, and hydrocarbons were found to be unidirectional and approximately equal in magnitude for variables condensed in the major discriminant root. This suggests a synergistic mechanism of action, which has been previously proposed for complex mineral water compositions [1,5]. The presence of salts in HASA, primarily NaCl along with trace elements, somewhat mitigated the stress manifestations, while the combination of inorganic and organic substances in native Hertsa water demonstrated a more pronounced stress-limiting effect.

Neuro-Endocrine-Immune Complex Interactions

Our canonical correlation analysis revealed that hormonal variables (PTH, aldosterone, testosterone, and markers of adrenal medullary function) determine metabolic and immune parameters by 81.3% (first pair) and 74.2% (second pair), respectively. These findings strongly support the concept of the neuro-endocrine-immune complex as an integrated regulatory system [1,5,20]. The factor structure of the hormonal roots indicates that chronic aversive stress factors cause the release of aldosterone, testosterone, and catecholamines from different layers of the adrenal glands into the blood, while simultaneously inhibiting PTH release.

The intake of significant amounts of NaCl prevented stress-induced aldosterone release and significantly reduced the release of catecholamines and testosterone, without affecting PTH release. This electrolyte-mediated hormonal modulation is consistent with the functional-metabolic continuum concept proposed by Gozhenko [21] and supports the role of mineral composition in balneotherapeutic effects [1].

Glomerular Filtration Rate Enhancement

A particularly noteworthy finding was the pronounced increase in glomerular filtration rate (GFR) caused by the intake of NaCl [22], which was maintained even with the addition of organic substances in native Hertsa water. This effect represents a significant physiological response that may contribute to the detoxification and metabolic benefits of mineral water therapy. The GFR enhancement aligns with Gozhenko et al.'s [22] research on renal functional reserve and suggests that mineral water intake may optimize kidney function beyond baseline levels.

Role of Organic Substances

While we were unable to identify individual classes of organic substances in native Hertsa water, the observed effects suggest compositional similarities to Naftussya bioactive water and Ukrainian phytocompositions [1,5,23]. The organic substances prevented catecholamine release and reduced stress-induced changes in blood levels of PTH and testosterone, though not aldosterone. Based on structural analogues, these organic compounds likely act through cortisol, testosterone, catecholamine, and polyunsaturated fatty acid receptors [24].

Recent research has highlighted the role of the aryl hydrocarbon receptor (AhR) in mediating the effects of phytoadaptogens [25-27]. Panossian et al. [24] proposed that adaptogenic compounds, including those found in mineral waters, may function through multiple receptor systems. Tang et al. [26] and Zhang et al. [27] demonstrated that polyphenolic compounds and ginsenosides activate the AhR/MAPK pathway, modulating immune function and tryptophan metabolism through gut microbiota interactions. This mechanism may explain the immunomodulatory effects observed in our study, particularly the changes in NK lymphocyte levels and neutrophil bactericidal capacity.

Comparison with Other Adaptogenic Interventions

The adaptogenic effects of Hertsa mineral water components are comparable to those documented for other Ukrainian phytocompositions. Studies on "Balm Truskavets'" [5,31-42] and ATINE [23,43-45] have demonstrated similar stress-limiting and immunomodulatory properties in both experimental animals and human subjects. Popovych [28,29] reported striking similarities between the effects of Naftussya bioactive water and phytocomposition "Balm Truskavets'," suggesting common mechanisms of action related to organic substance content.

Fihura et al. [32-42] conducted extensive research on "Balm Truskavets'" demonstrating its ability to modulate EEG, HRV, and biophotonics parameters, as well as to ameliorate the effects of balneofactors in patients with various conditions including post-radiation encephalopathy. These findings support the concept that organic substances in mineral waters and phytocompositions share adaptogenic properties that can be therapeutically exploited.

Metabolic and Immune Implications

The observed changes in electrolyte metabolism, particularly the reversal of stress-induced hyponatremia into hypernatremia and the leveling of decreased adrenal medullary zone thickness by both native Hertsa water and HASA, indicate complex regulatory mechanisms. The slight deepening of hypokalemia by both preparations suggests that potassium supplementation might be considered during prolonged mineral water therapy.

The immunomodulatory effects, including changes in NK lymphocyte levels, neutrophil phagocytic activity, and bactericidal capacity, demonstrate the immune-regulatory potential of mineral water components. These effects are consistent with the immune-neuro-endocrine interactions described by Besedovsky and del Rey [20] and support the therapeutic application of mineral waters in conditions requiring immune system modulation [12,13].

Methodological Considerations

The use of discriminant analysis and canonical correlation analysis in our study allowed for the quantitative assessment of the contributions of different mineral water components. The calculation of Shannon's entropy for various cytograms (leukocytogram, immunocytogram, thymocytogram, and splenocytogram) [13-16] provided a comprehensive measure of immune system organization and complexity. This methodological approach, developed by the

Truskavetsian Scientific School of Balneology and Phytotherapy [1,5,18], enables a more nuanced understanding of the multifaceted effects of balneotherapeutic interventions.

Clinical Implications and Future Directions

The unique combination of salts, trace elements, and organic substances in Hertsa drinking mineral water suggests its potential therapeutic application in stress-related disorders and conditions requiring metabolic and immune system support. The quantitative assessment of individual component contributions provides a scientific basis for optimizing mineral water formulations and treatment protocols.

Future research should focus on:

Identifying specific classes of organic substances in Hertsa water and their individual contributions to physiological effects

Investigating the molecular mechanisms of AhR activation by mineral water organic components

Conducting clinical trials to evaluate the therapeutic efficacy of Hertsa water in specific patient populations

Exploring the optimal dosing regimens and treatment durations for different clinical conditions

Examining potential interactions between mineral water components and conventional medications

Limitations

This study has several limitations. First, the relatively small sample size (n=10 per group) may limit the generalizability of findings. Second, the inability to identify specific organic substance classes prevents a more detailed mechanistic analysis. Third, the study was conducted exclusively in female rats, and sex-specific differences in response to mineral water components may exist. Finally, the acute experimental protocol (6-day treatment) may not fully reflect the effects of longer-term mineral water consumption.

Conclusion

In conclusion, our study demonstrates that both inorganic and organic components of Hertsa drinking mineral water contribute significantly to its effects on the neuro-endocrine-immune complex and metabolism in female rats. The stress-limiting, metabolic, and immunomodulatory properties of this water support its potential therapeutic application. The quantitative assessment of component contributions provides valuable insights for understanding the mechanisms of balneotherapy and optimizing mineral water-based interventions.

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Declarations

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Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets used and/or analyzed during the current study are open from the corresponding author Kovalchuk G.Y. on reasonable request.

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