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	PROJECT: **WILDLIVE AND BIODIVERSITY MONITORING IN ATYRAU AND MANGYSTAU REGIONS**	
	CONTRACTORS: **KAZAKHSTAN AGENCY OF APPLIED ECOLOGY (KAPE)** **A.N. SEVERTSOV INSTITUTE OF ECOLOGY AND EVOLUTION OF THE RUSSIAN ACADEMY OF SCIENCES (IEE RAS)**	

SURVEY OF THE CASPIAN SEAL IN THE WATER AREA OF THE NORTH CASPIAN SEA. AUTUMN, 2023

Section 2: Survey of the Caspian Seal Population as an Endemic of the Caspian Sea Fauna and Indicator of the Caspian Sea Ecosystem

REPORT

REVISION 01

ABSTRACT

The work was carried out in accordance with Contract UI176768.

The report includes data on the Caspian Seal population in the North Caspian Sea. All surveys of the Caspian Sea were conducted using non-invasive and minimally invasive methods, which did not cause significant harm to individual animals or the population as a whole.

Biological studies were conducted by specialists from Kazakhstan (RPC VM) and Russia (IEE RAS).

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1. INTRODUCTION

Monitoring the status of marine mammal populations is one of the most important areas of environmental surveys as an integral component of wildlife conservation strategies and control of potential threats to human and animal health. Marine mammals in many areas are used as indicator species for medium- and long-term observations of changes in the external environment [Aguilar, Borrell, 1994].

The Caspian seal (*Pusa caspica*, Gmelin, 1788) is the only marine mammal species inhabiting the Caspian Sea. Endemic, it plays a unique role in the Caspian ecosystem, being the closing link in the trophic chain of the Caspian aquatic ecosystem, inhabits the entire sea area and can be considered an indicator species of its condition.

Since 2008, the Caspian seal has had Endangered conservation status in the Red List of the International Union for Conservation of Nature. In 2017, more than 100 countries classified the Caspian seal as Endangered. According to the UN resolution adopted within the framework of the Convention on the Conservation of Migratory Species of Wild Animals (Bonn Convention) at the 12th session of the Conference of the Parties, the Caspian seal is included in Annexes I and II to the Convention [Annexes I et II de la Convention, 2018]. This implies the necessity of a ban on fishing for the seal and obliges Parties to the Convention to make maximum efforts to conserve and restore the population, protect habitats, remove obstacles to migration, and mitigate the impact of unfavourable factors [Convention, 2003].

The Caspian seal's habitat is located in five Caspian littoral states: Russia, Kazakhstan, Azerbaijan, Turkmenistan and Iran. In 1993, the species was included in the Red Book of Azerbaijan, in 2011 in the Red Book of Turkmenistan. - The species was included in the Red Data Book of Turkmenistan in 2011. Iran has adopted the Endangered conservation status defined by the International Union for Conservation of Nature. In 2020, the Caspian seal was included in the Red Books of the Russian Federation and the Republic of Kazakhstan.

According to an international team of researchers from Kazakhstan, Russia and European countries, who conducted visual aerial surveys in the Kazakhstan sea sector, the number of the species in the Caspian Sea has decreased from 1.2 million specimens to 168 thousand - by 86% over 105 years (from 1900 to 2005) [Harkonen et al., 2012]. According to the data of visual and multispectral aerial surveys in 2012 and 2022, carried out by Russian, Kazakh and British specialists, the estimated number of seals in the waters of the North Caspian Sea was 270 - 311 thousand specimens [Kuznetsov et al., 2013; KAPE, VNIRO, 2022].

The main critical threats to the Caspian seal are: bycatch in illegal fishing, as well as the growth of illegal seal harvesting (intentional fishing with sturgeon fishing gear and illegal capture), which is stimulated by the increased market demand for seal skins and fat both in the markets of the Caspian Sea and in other regions.

Disturbance factor and habitat degradation are also considered to be serious threats. Moderate threats include Caspian seal diseases, including mass epizootics, parasitic infestations, bioaccumulation of toxicants causing immunosuppression, pathomorphological disturbance of internal organs, including reproductive organs. Potential threats that require attention, but are currently unappreciated in terms of their impact on the population, should be considered as: the impact of pollution on the food chain; overfishing of food objects (fish); spread of invasive species; degradation/loss of habitats of those fish species that form the basis of the seal diet; climate change causing reduction of ice fields (seal breeding grounds) and excessive heating of surface waters of the Caspian Sea in summer.

Russian and Kazakh territorial waters of the Caspian Sea, especially the northern part (North Caspian Sea), are the most important area for the Caspian seal. From autumn to mid-spring, the main part of the population, migrating across the sea, aggregates in the North Caspian Sea for one of the most important stages of the life cycle - breeding. At the same time, Kazakhstan and Russian territorial waters are an area of growing anthropogenic impact on the marine ecosystem, which affects the well-being of the Caspian seal population.

Currently, not everything is known about the biology and ecology of the Caspian seal.

There is no stable information of the total number of animals, migration routes and peculiarities of water area use, including changes in movements depending on the impact of environmental factors, are poorly studied. Circulation of various diseases in the population has not been studied and the level of immunity of the animals has not been assessed. There is no information of their hormonal status and no idea of the genetic structure of the species. There is no reliable non-invasive way to determine the age of seals. All this information is necessary for the development of adequate and effective measures for the protection of this species.

In order to ensure environmental protection, biodiversity conservation in terms of the Caspian seal and its key habitats in the Caspian Sea, within the jurisdiction of the Russian Federation and the Republic of Kazakhstan, the "Programme of Surveys of the Caspian Seal in the Waters of the North Caspian Sea for 2019-2023" has been approved. This Survey Programme has been developed taking into account the conditions and requirements of the Convention on the Legal Status of the Caspian Sea, signed on August 12, 2011.

The Programme is based on the principles set out in the Convention for the protection of the natural environment of the Caspian Sea - conservation, restoration and rational use of its biological resources and promotion of scientific researches in the field of ecology, conservation and use of biological resources of the Caspian Sea.

Currently, not everything is known about the biology and ecology of the Caspian seal. There is no information of the exact number of animals, their migration routes and peculiarities of water area use, including changes in movements depending on the impact of environmental factors, are poorly known. Circulation of various diseases in the population has not been studied and the level of immunity of the animals has not been assessed. There is no information of their hormonal status and no idea of the genetic structure of the species. The causative agents of viral diseases of marine mammals are identified only after mass outbreaks of infections that have reached epizootic proportions. Hence, there is a need for proactive diagnostic methods for the timely identification of novel viruses with zoonotic potential to cause disease outbreaks

in order to develop an effective strategy to control devastating epizootics of vulnerable animal species. Diseases of bacterial nature can be either independent, secondary or opportunistic infections. In this regard, it is important to study both normal, conditionally pathogenic and pathogenic microflora in Caspian seal, which will enable to evaluate their role in the overall pathology of mass outbreaks of infectious diseases.

In spring 2000, the morbillivirus etiology of the Caspian seal mass mortality was established [Kennedy et al., 2000, Kuiken et al., 2000]. The evolution of morbillivirus in Caspian seal in 2000 was studied by recovering full-length genome sequences from archival materials using next-generation sequencing [Jo et al., 2019]. In recent years, the Caspian seal herpesvirus has been identified through studies using modern molecular diagnostic techniques. The seal herpesvirus PhHV-1 was detected in samples collected in 2016. [Kydyrmanov et al., 2019].

Using the mass parallel sequencing method, the composition of viral metapopulations of Caspian seal viruses has been preliminarily studied by the staff of the virus ecology laboratory of the Microbiology and Virology Research Centre, and contigs of viruses belonging to the families *Orthomyxoviridae, Paramyxoviridae, Pneumoviridae, Flaviviridae, Herpesviridae,* and *Papillomaviridae* have been identified. In recent 2023 studies, three new Caspian seal viruses have been identified and tentatively categorized as parvovirus-like (*seal parvovirus type*), annellvirus-like (*Seal anellovirus type*) and circovirus-like (*Fur seal circovirus type*) viruses.

In January 2023, specialists from the Republic of Kazakhstan subjected tissue samples collected from fallen seals to PCR screening on the Dagestan coast of the Caspian Sea. As a result of OT-PCR with primers to the P-gene of morbilliviruses (plague of carnivores), the expected products of 429 bp were detected in lung tissues of 34 seals. A live modified vaccine to carnivore plague virus (Pfizer, USA) was used to control specificity. To determine the infection of seals with orthomyxoviruses, OT-PCR with primers to the M-gene of influenza A (H5N1) virus was performed. As a result of electrotorphoresis, in 2% agarose gel, the expected PCR products of 279 bp were detected in the lung samples of nine seals. Thus, in the examined samples of Caspian seals from the Dagestan coast of the sea, in winter 2023, the causative agents of influenza and morbillivirus infections in dead seals were detected simultaneously.

In autumn 2023, research work was carried out in accordance with Contract UI176768, Purchase Order No. 4512583596 and No. 4512585696. The completed Scope of Work is a part of the above-mentioned Programme (**Appendix A**).

1.1. Goal and Objectives of Surveys

Survey goal: To assess the abundance, distribution and well-being of the Caspian seal population in the North Caspian Sea water area in 2023.

Survey objectives:

- Capture of seals, performance of morphometric studies, selection of biological material for toxicological, physiological (serological and hormonal), virological and microscopic studies in line with the Programme "Surveys of Caspian Seal in the North Caspian Sea Water Area in 2019-2023" approved by the Company dated 03 July 2019;
- studying the movement routes of Caspian seals using satellite telemetry by installing at least 10 satellite beacons on captured animals in the waters of the North Caspian Sea during the spring period;
- counting the number of seals on vessel routes, rookeries, and field work sites in autumn;
- development of proposals on population conservation.

1.2. Dissemination of the Document and Target Audience

Unless otherwise authorized by NCOC N.V., this document shall be for NCOC N.V. internal use.

1.3. Definitions, Abbreviations and Acronyms

1.3.1. General Definitions

Company shall mean North Caspian Operating Company N.V. (NCOC N.V.).

"**Contractor**" shall mean the party providing services to the Company during the term of the Contract on a Purchase Order basis, including project management and preparatory work, field survey work, analytical work, and reporting.

"**Supplier**" (**Manufacturer/Supplier**) shall mean the party that manufactures or supplies equipment and services to fulfil the obligations specified by the Contractor.

The word "**shall**" means that a provision is enforceable.

The word "**should**" shall means that a provision is not mandatory but is recommended as a good work practice.

1.3.2. Special Terms, Definitions, Abbreviations and Acronyms

List of abbreviations:

Term / Acronym / Abbreviation / Definition	Interpretation / definition
AIS	Automatic Identification System for tracking the movement of ships
BLAST	Basic Local Alignment Search Tool
E	East longitude
GPS	Global Positioning System
HPAI- viruses	Highly pathogenic avian influenza
LC50	50 per cent lethal concentration
LPAI- viruses	Low pathogenic avian influenza
N	Northern latitude
N (NA)	Neuraminidase
NMDA	N-methyl-D-aspartate (NMDA) glutamate receptors
PhHV-1	Herpesvirus type 1
TCPMe	Tris-(4-chlorophenyl)-methane
TCPMOH	Tris-(4-chlorophenyl)-methanol
TEQ	Toxic equivalent

Term / Acronym / Abbreviation / Definition	Interpretation / definition
ZcAV	California sea lion annelovirus
PP	Petroleum products
TCDD	Tetrachlorodibenzo-p-dioxin
AH	Aromatic hydrocarbons
BF	Bioaccumulation factor
BOD	Biochemical oxygen demand
UAV	Unmanned aerial vehicle
IV	Influenza virus
IAV	Influenza A virus
AIV	Avian influenza virus
WHO	World Health Organisation
CPV	Carnivore plague virus
HBCD	Hexabromocyclododecane
GC/MS	Combination of gas chromatography and mass spectrometry
HCB	Hexachlorobenzene
HCCH	Hexachlorocyclohexane
DDD	Dichlorodiphenyldichloroethylene
DDT	1,1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane)
RSE	Remote sensing of the Earth
DNA	Deoxyribonucleic acid
AL	Acceptable level
IIWB RAS	Federal State Budgetary Institution of Science “I.D. Papanin Institute of Inland Waters Biology” of the Russian Academy of Sciences
KAPE CAC TL	Testing Laboratory of the Chemical and Analytical Centre of Kazakhstan Agency of Applied Ecology LLC
IEEP RAS	Federal State Budgetary Institution of Science “A.N. Severtsov Institute of Ecology and Evolution Problems” of the Russian Academy of Sciences.
IEA	Immunoenzymatic analysis
Cdna	Complementary deoxyribonucleic acid
KSCS	Kazakhstan sector of the Caspian Sea
RF RDB	Red Data Book of the Russian Federation
MM	Marine mammals
SB	Seabirds
IUCN	International Union for Conservation of Nature
H (HA)	Haemagglutinin
R/V	Research vessel
HSES	Health, Safety, Labour, Environment and Security
ARVI	Acute respiratory viral infections
RT-PCR	Reverse transcription-polymerase chain reaction
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ethers
MPC	Maximum permissible concentration
p.b.	Paired base
PCB	Polychlorinated biphenyls
PCDD/PCDF	Polychlorinated dibenzo-p-dioxins and Polychlorinated dibenzofurans
PCR	Polymerase chain reaction
HGR	Haemagglutination reaction
RoK	Republic of Kazakhstan
RNA	Ribonucleic acid
Rrna	Ribosomal ribonucleic acid
HGIR	Haemagglutination inhibition reaction
RF	Russian Federation
SEM	Scanning electron microscopy
PPE	Personal protection equipment
NCMC	North Caspian Marine Channel
POP	Persistent organic pollutants
SSAS	Synthetic surfactants
HM	Heavy metals
KAPE LLC (KAPE)	Kazakhstan Agency of Applied Ecology, Limited Liability Company
SPC M&V LLP	Scientific and Production Centre of Microbiology and Virology, Limited Liability Partnership
HC	Hydrocarbons

Term / Acronym / Abbreviation / Definition	Interpretation / definition
FSBSI "RRIFO" (VNIRO)	Federal State Budgetary Scientific Institution "All-Russian Research Institute of Fishery and Oceanography"
FSBI "FRC FTM"	Federal State Budgetary Scientific Institution "Federal Research Centre for Fundamental and Translational Medicine"
FSBEI HE "ASTU"	Federal State Budgetary Educational Institution of Higher Education "Astrakhan State Technical University"
FSBI "TSPA"	Federal State Budgetary Institution "Typhoon Scientific and Production Association"
BFE	Blood Form Elements
OCC	Organochlorine compounds
COD	Chemical oxygen demand
A	Alive - live Caspian seal
AF	Aborted Foetus
AIS	Automatic Identification System - Automatic Identification System for tracking the movement of ships
BSE	Back-reflected, or back-scattered electrons mode
Ca	Calcium
CV	Coefficient of variation
D	Dead Caspian seal
ECM	Extracellular matrix
Fe	Iron
GPS	Global Positioning System
Hg	Mercury
Mg	Magnesium
NCOC N.V.	North Caspian Operating Company N.V.
Na	Sodium
P	Phosphorus
SD	Standard deviation
SSE	Secondary reflected electrons mode
Wn	Range of values in the sample with the number of components (n)
WoS	Web of Science) - polythematic abstract-bibliographic and bibliometric database on world scientific publications
O	Oxygen
C	Carbon

List of definitions:

Term / Abbreviation/Acronym	Interpretation / definition
A/Fowl Plague/Dutch/27 (H7N7)	the first isolated and studied strain of influenza A virus of subtype H7N7 (in 1927). This strain was part of the diagnostic serum panel used in the USA.
AGID (Agar Gel Immunodiffusion)	Immunodiffusion in agar gel. In Russian literature, the precipitation reaction in agar gel. For agar gel immunodiffusion tests (AGID, gel ID or double diffusion), round wells are cut in the agar layer on a plastic plate using a punch. One well is usually the central well, is filled with soluble antigen and the other wells are filled with the test serum. A positive control antibody sample is included in each assay. The reagents are allowed to diffuse into the agar and when antibody and antigen meet in optimal proportions (equivalence point), immune complexes are formed. This results in a visible precipitate line between the antigen well and the wells containing positive serum
BLAST – Basic Local Alignment Search Tool	A family of computer programs used to search for homologues of proteins or genes for which the primary structure (sequence) or a fragment thereof is known. BLAST can be used to compare an available sequence with sequences from a database and find similar or deleted proteins/genes. a newborn unmoulted seal pup [Geptner et al., 1976]. An analytical method used to identify specific proteins in a sample. In the first step, protein electrophoresis in a polyacrylamide gel is used to separate the denatured proteins
Acanthocephales (proboscis worms, acanthocephalous)	belong to the *Acanthocephales* type, *Acanthocephala* class.
Baby seal	a newborn unmoulted seal pup [Geptner et al., 1976].
Blot analysis, Western blotting (Western blot, protein immunoblot)	An analytical method used to identify specific proteins in a sample. The first step uses protein electrophoresis in a polyacrylamide gel to separate denatured proteins by length or three-dimensional structure. Next, the proteins are transferred onto a nitrocellulose or PVDF membrane, then are detected using antibodies specific to a given protein. There are many commercial companies specialising in producing antibodies to tens of thousands of different proteins.

Term / Abbreviation/Acronym	Interpretation / definition
Yearling	A Caspian seal between 1 and 2 years old.
Vaccine strain	a strain of microorganism used in different types of vaccination (as a vaccine)
Helminths	represent an environmental group indicating the way of life and the harm they cause to various living organisms. The concept of helminths is limited to parasitic organisms of the Platheminthes types (flatworms), Nemathelminthes (roundworms) and Acanthocephales (proboscis worms)
Gram-positive bacteria	Bacteria that do not have an outer additional membrane of lipopolysaccharides and are stained purple when using Gram staining of microorganisms.
Graticules	grid of latitude and longitude in cartograms
Definitive (final) host	organism in which sexual reproduction of the parasite takes place
Dynamics of change	quantitative value is assessed through linear trend (slope coefficient of the regression line). Qualitative value (positive dynamics, negative dynamics) - through the sign (plus, minus) of the slope coefficient of the regression straight line
Change significance	quantitative value is assessed through the statistical significance of the linear trend. Qualitative - the significance of changes is positive when the statistical significance level exceeds 0.95 (unless another significance level is specified). Figures are displayed with the sign of the dynamics of changes (positive, negative) to improve perception
Isolate	In microbiology, an isolate is a culture of viruses or other microbes isolated from a specific source.
Invasion intensity (II)	The number of parasites found in the examined animal, expressed in specimens.
Enzyme immunoassay (EIA)	enzyme-linked immunosorbent assay (ELISA) is a laboratory immunological method for qualitative or quantitative determination of various compounds, macromolecules, viruses, etc., based on a specific antigen-antibody reaction. The detection of the formed complex is carried out using an enzyme as a tag to record the signal. ELISA is unique due to the specificity of the immunochemical reaction (i.e. antibodies bind exclusively to certain antigens and no others).
Keratinised skin derivatives	wool, vibrissum
Interannual variability	for the selected month or season, the range of values (standard deviation from the mean annual value).
Nematodes	round helminths belonging to the Nemathelminthes type genus, Nematoda class
Pagetode (pagophilous) seal species	species that use ice for birth, nursing, moulting, mating and resting [Geptner et al., 1976].
PES	practical salinity unit - a unit of measurement of salinity
Air hole	rounded holes in the ice cover, made and used by seals for breathing [Geptner et al., 1976].
Pseudamphistomum truncatum	A parasitic disease from the group of trematodoses, the causative agent of which is the trematode *Pseudamphistomum truncatum* (Opisthorchidae family), [Akbaev, Esaulova, 2004].
HAIR	Haemagglutination inhibition reaction. A method of virus identification or detection of antiviral antibodies in blood serum based on the phenomenon of absence of agglutination of erythrocytes by a preparation containing virus in the presence of serum immune to it. HAIR is based on blockade, suppression of virus antigens by antibodies of immune serum, as a result of which viruses lose their ability to agglutinate erythrocytes. HAIE is used to diagnose many viral diseases, the causative agents of which can agglutinate erythrocytes of various animals
Young-of-the-year	Caspian seal under 1 year of age
Sivar	a Caspian seal pup that has finally moulted [Geptner et al., 1976].
Average arithmetic	a number equal to the sum of all numbers of a set divided by their number. Used to calculate an estimate of the mathematical expectation. It is used to estimate the mean annual value obtained for the selected season or month of the year from the available multi-year data set
Standard deviation, σ	an index of the dispersion of values of a random variable relative to its mathematical expectation. The square root of the sum of the squares of the deviations from the mean divided by the number of measurements. The standard deviation is usually mentioned to indicate the scatter after the mean value; notation may be given to shorten the record
Annual average value	for the selected month or season, the mean (arithmetic) value for the period under consideration
Standard error trend	an estimate of the error of the slope coefficient of a linear regression straight line
Statistical trend significance	a dimensionless value characterising the reliability of the obtained estimate of the slope coefficient of the regression line.
Trematodes	are flat helminths of the PlathemInthes type, Trematoda class (suckers).
Trend, linear trend	the change in the analysed parameter over the period under consideration. It is used to estimate the slope coefficient of the linear regression straight line. Used to assess the dynamics of change
Cestodes	tapeworms belonging to the PlathemInthes type, Cestoda class
Sand islands	a typical offshore shallow island in the Caspian Sea, formed naturally on shallow banks of sand and small shell sediments by easterly winds (local name).
Strain	A lower infrasubspecies systematic category denoting an identified pure culture of microorganisms of the same species in which morphological and physiological features were studied.

Term / Abbreviation/Acronym	Interpretation / definition
Invasion intensity (IE)	the ratio of the number of infected animals to the total number of animals examined, expressed as a percentage.
Essential chemical elements	the elements without which an organism cannot grow, develop and fulfil its life cycle.
Infertility	A term that refers to the under-receipt of calves in a breeding herd during the past year due to abortion or infertility. It is expressed as a percentage (if 80 calves from 100 mothers are produced in the mother group of a herd, the infertility is 20%), [Veterinary Encyclopaedic Dictionary, 1981].

1.4. Reference Documents and links

Ref. No..	Document No. / Reference	Title / Description
(1)	No. 400-VI g. Of 02.01.2021 Chapter 16, Article 245, 256; Chapter 19, Article 269, 274.	Environmental Code of the Republic of Kazakhstan
(2)	No. 1469 of December 31, 2004	Decree of the Government of the Republic of Kazakhstan . On approval of the Rules for issuing permits for the use of wildlife
(3)	No. 652 of November 10, 2004	Order of the Minister of Agriculture of the Republic of Kazakhstan . On approval of the Rules for the use of animals, except rare and endangered ones, for scientific, cultural, educational and aesthetic purposes, including for the creation of zoological collections.
(4)	Marine Mammal Necropsy: An introductory guide for stranding responders and field biologists. Woods Hole Oceanog. Inst. Tech.Rept. WHOI-2007-06.	Marine Mammal Necropsy: An introductory guide for stranding responders and field biologist
(5)	Office International des Epizooties (OIE), Manual of standards for diagnostic tests and vaccines. – Paris, 2000	Manual of standards for diagnostic tests and vaccines.
(6)	TITLE IV, Marine Mammal Health and Stranding Response. – 1972	The Marine Mammal Protection Act of 1972 As Amended
(7)	WHO/CDS/CSR/NCS/ Manual for on Animal Influenza Diagnosis and Surveillance/ - Geneva, 2002. – P. 15-18.	Manual for on Animal Influenza Diagnosis and Surveillance
(8)	CRC Handbook of Marine Mammal Medicine, Boca Raton, Florida: CRC Press, 2018, 3rd ed.	CRC Handbook of Marine Mammal Medicine

2. ROLES AND RESPONSIBILITIES

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Ibrayev S.	Senior Specialist	KAPE	Almaty
Abdusamatova B.	Senior Specialist	KAPE	Almaty
Rakhimov E.A.	Senior Specialist	KAPE	Almaty
Zhanatova A.J.	Senior Specialist	KAPE	Almaty
Kaldaev S.	Senior Specialist	KAPE	Almaty
Mishchenko V.P.	Senior Specialist	KAPE	Almaty
Orlova I.V.	Senior Specialist	KAPE	Almaty
Sapozhnokova I.A.	Senior Specialist	KAPE	Almaty
Fomina E.	Senior Specialist	KAPE	Almaty
Chernov A.M.	Head of GIS Department	KAPE	Almaty
Kydirmanov A.I.	Specialist - Virologist	SPC M&V	Almaty
Karamendin K.O.	Specialist - Parasitologist	SPC M&V	Almaty
Kasymbekov E.T.	Research Associate	SPC M&V	Almaty
Sabyrzhan T.	Junior Research Fellow	SPC M&V	Almaty
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Solomentseva S.M.	Head of TA Unit	KAPE	Almaty
Danilenko V.V.	Translator	KAPE	Almaty
Otarbayeva A.	Translator	KAPE	Almaty
Baisartova A.	Translator	KAPE	Almaty
Vessel “Nautilus-One”			
Barsukov S.I.	Captain	KAPE	Kazakhstan, Bautinon
Kulikov A.E.	Senior Mate of the Captain	KAPE	Kazakhstan, Aktau
Konischev S.K.	Senior Mechanic	KAPE	Russia, Astrakhan
Auganov A.S.	Second Mechanic	KAPE	Kazakhstan, Fort-Shevchenko
Izturgan A.	Bosun	KAPE	Kazakhstan, Bautino
Koval K.Yu.	Sailor	KAPE	Kazakhstan, Bautino
Nurzhanova K.S.	Cook	KAPE	Kazakhstan, Aktau

RUSSIA:

Personnel	Position / Role	Institute/Company (employment)	Location
Scientific staff			
Naidenko S.V.	Director, Ph.D. in Biology	IEEP RAS	Moscow
Rozhnov V.V.	Head of the Laboratory, Academician of the Russian Academy of Sciences, Ph.D. in Biology	IEEP RAS	Moscow
Solovieva M.A.	Project Manager, Senior Researcher, Candidate of Biological Sciences	IEEP RAS	Moscow
Surov A.V.	Chief Scientist, Deputy Director for Science, Ph.D. in Biology	IEEP RAS	Moscow
Glazov D.M.	Lead Engineer	IEEP RAS	Moscow
Vecherskiy M.V..	Senior Researcher, Candidate of Biological Sciences	IEEP RAS	Moscow
Platonov N.G.	Senior Researcher	IEEP RAS	Moscow
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Shibanova P.Y.	Junior Research Fellow	IEEP RAS	Moscow
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Pilipenko G.Yu.	Laboratory Technician	IEEP RAS	Moscow
Udodenko Yu.G.	Chief Scientist	IEEP (IIWB) RAS	Moscow
Komov V.T.	Chief Scientist, Ph.D. in Biology	IEEP (IIWB) RAS	Moscow

3. FIELD WORK PROGRAMME

3.1. Survey Plan

The main objective of the autumn 2023 surveys was to study the current status of the Caspian seal population as an endemic of the Caspian Sea fauna and an indicator species of the Caspian ecosystem status.

In order to study the current status of the Caspian seal population, the seals were captured in the autumn of 2023 at the KSCS sea sand island.

A DJI Mavic 2 Pro quadrocopter was used to count the number of Caspian seals on islands and sand islans, search for approaches to areas with resting animals for landing of scientists in areas where animals aggregate. This survey allowed to estimate the number of seals and to determine the location of the boat approach to catch seals.

In the area from the port of Bautino to the north-eastern part of the Seal Islands (Kulaly Island) and from the Saddle Island area to the NCMC, observations were made to count the number of Caspian seals from the running bridge of the R/V Nautilus-One. Counting was carried out under favourable weather conditions during daylight hours (4-hour shifts). Observations and counting were carried out visually by one to three observers using Nikon 10x50 binoculars along the vessel's course.

The demographic structure of the Caspian seal population (the ratio of fathers and mothers of different ages) was studied in parallel with sampling of their genetic material and other samples at the seal rookeries site. Kazakhstan and Russian specialists collected biological material from captured specimens using certified methods recommended by the International Epizootic Bureau and the Marine Mammal Commission.

3.2. Timing and Area of Surveys

In the Kazakhstan sector of the North Caspian Sea, field surveys were conducted from 06rh to 17th of November 2023 as part of a joint expedition of KAPE (4 specialists), IEEP RAS (4 specialists) and SPC M&V (4 specialists) at the R/V Nautilus-One. Seals were counted during the vessel's movement from Bautino port to the Kulaly Island area and the North Caspian Marine Channel area.

Figure 3.2.1 Nautilus One Research Vessel

Before the start of the expedition, a large release of dead seals was reported on the shore of Tyup-Karagan Spit and also on the coast of Bautino settlement. Bautino settlement. On 5th of November, a survey of dead seals in the amount of 40 specimens was conducted.

Vessel routes are presented in Figure 3.2.2.

Figure 3.2.2 Vessel and Science Team Routes in November 2023

3.3. Methodology of Field Work

3.3.1. Caspian Seal Census

Field work on the surveys includes shore-based and vessel-based observations.

Shore-based observations. Observations are made from one or more observation points located within walking distance of the detected rookery, but at a distance so as not to disturb burrowing animals (from natural or artificial shelter, if necessary). It is possible to use prefabricated structures as observation posts. Observation points can be shifted according to the information obtained during fieldwork and the local topography.

Observers use two main methods:

– Horizon scanning to obtain estimates of relative population density and the size of seal groups on the coast. Binoculars with 10-12x magnification are used. Scanning is carried out in mode - 1 time intervals during daylight hours and under favourable weather conditions. Time intervals for scanning are selected based on animal behaviour and dynamics of their movement;

– continuous observation of the water area in order to correct the scanning results, as well as observations of certain speciments or groups of seals in order to describe the behaviour on the surface.

Shipboard visual observations in the water area are carried out from a direction finding platform or other elevated position at the vessel, including from the bridge, in unfavourable weather, as well as from a small vessel.

It should be taken into account that under the best observation conditions the visibility (distance to the horizon line) will be about 10 km, if the observer is viewing the water area with binoculars at a height of about 7 m from the sea surface. Seal counts were conducted within a radius of up to 500 m from the vessel, and 300 m from the workboat.

Observations are conducted throughout the daylight hours by two observers in a team of 4 people. In the dark, observations may be made while the vessel is moored or passing close to shore.

The maximum viewing angles from the direction finding platform are wide enough - 120^0 to the left and 120^0 to the right of the vessel"s course, from the bridge the view is limited by the portholes, including the range and clarity is significantly affected by the “noise” of the porthole glass. From the workboat, the viewing angle is 90 degrees.

Observations from the bridge shall be made only in bad weather conditions, which primarily affect the quality of observations and may damage the equipment used (low temperatures, heavy rain, rocking of the vessel, etc.). From a workboat, in bad weather conditions (in swell over 50 cm and wind speed over 8-10 m/s), seal counting is not carried out.

During the voyage of the R/V Nautilus-One from 3^{rd} to 8^{th} of November 2022, the seal count took place when the vessel was moving from Bautino towards the seal islands in the Kazakhstan water area of the Caspian Sea.

Counting of the number of seals on sand islands in the Kazakhstan sector of the Caspian Sea was carried out using a quadrocopter DJI Mavic 2 Pro. The specifications of the quadrocopter are listed in Table 3.3-1.

The UAV was controlled by one operator, who also took photos and video recordings of animals during the flight. During the aerial survey, continuous video recording was carried out using a standard video camera. Take-off was performed from a motorboat. The flight altitude varied from 80 to 100 metres from the water and depended on the route and purpose of the copter overflight.

During the observations, all current information was recorded on a voice recorder or in a notebook; coordinates were determined by a GPS receiver and duplicated by the vessel’s AIS system. All observations were recorded in specially designed electronic forms.

Table 3.3-1 Specifications of DJI Mavic 2 Pro (UAV)

Equipment designation	Equipment specifications	Photo
DJI Mavic 2 Pro Quadrocopter	Maximum take-off speed - 6 m/s (Sport mode)/5 m/s (Position mode); Maximum descent speed - 3 m/s (Sport mode)/3 m/s (Position mode); Maximum horizontal flight speed - 72 km/h (Sport mode)/50 km/h (Position mode)/58 km/h (Atti mode); Maximum pitch angle - 42° (Sport mode); 25°/(Position mode); Maximum angular speed - 250°/s (Sport mode)/150°/s (Atti mode); Maximum flight altitude - 6000 m above sea level; Maximum flight altitude above take-off point - 500 m; Flight time - about 30 minutes; Permissible operating temperature - from 0°C to 40°C; Supported satellite navigation systems - GPS/GLONASS	

To obtain objective weather data, weather conditions are recorded regularly (once every 6 hours) and at changes in the form "Record of Meteorological Data During Observations". The following information is recorded: date; name of the observer; time; wind, direction (degrees); wind speed (m/s); cloudiness (points); air temperature (degrees C); precipitation; humidity (%); atmospheric pressure (hPa); swell (period in sec.); swell (amount, half metres); ice characteristic (cohesion); visibility (km); Beaufort (wind swell); vessel's course (degrees); vessel's speed (km/h); comments.

3.3.2. Caspian Seal Capture

Seals were captured by the expedition participants on sandy islands during daylight hours (Figure 3.3.1). To catch the animals, nets were used (Figure 3.3.2 a), which consisted of a steel hoop with a diameter of about 100 cm (wire thickness - 8 mm), a wooden handle with a length of 2 m and a 3 m long net (deli) attached to the hoop, made of 1.5 mm thick kapron thread with a mesh of 70-80 mm.

After capture, seals were placed in zip-lock tarpaulin bags (Figure 3.3.2 b) with ventilation holes. No medical preparations were used to immobilise the animals.

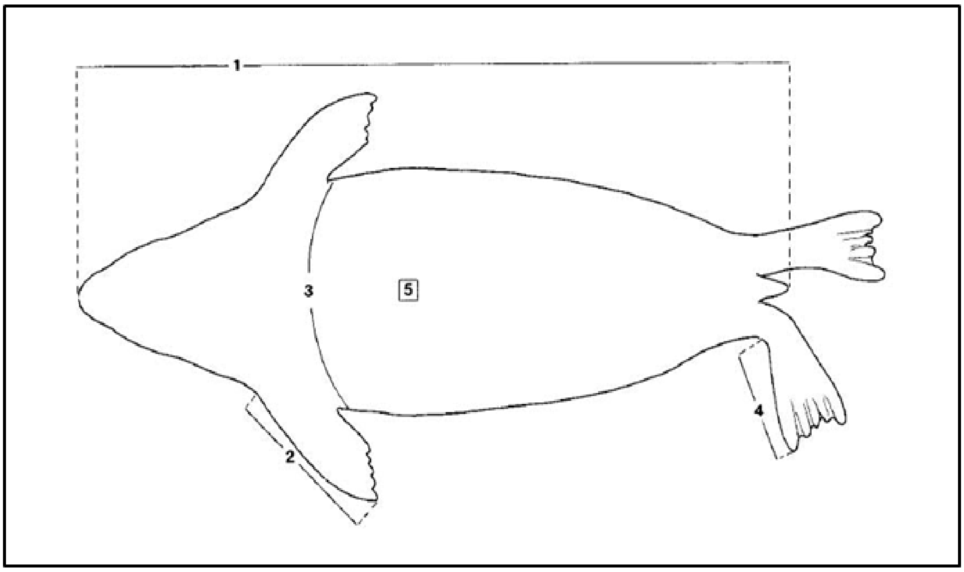
Figure 3.3.1 Seal Capture

a) Catch net **b) Transportation bag**

Figure 3.3.2 Tools for Ccapturing and Transporting Seals

3.3.3. Morphometric Studies

Zoological body length (curvilinear body length) - measured from the lower points of the nostrils to the end of the tail (the line runs along the head and back), (Figure 3.3.3).



1 –projection length; 2 - length of the anterior flipper; 3 - thorax girth;

4 - hind flipper length [Geraci, Lounsbury, 1998]

Figure 3.3.3 Measurement of the Seal

Projection zoological body length (standard body length) - distance from the lower points of the nostrils to the end of the tail of the animal in horizontal projection. Perpendicular to the body of an animal lying on its belly, two parallel lines are drawn along the surface on which it lies: one from the nostrils and the other from the end of the tail and the distance between them is measured.

Total projection body length is the distance from the nostrils to the end of the left hind flipper in horizontal projection. Perpendicular to the body of an animal lying on its belly, two parallel lines are drawn along the surface on which it lies: one from the nostrils, the other from the end of the left hind flipper (to the end of the first toe) and the distance between them is measured.

The length of the tail was measured from its base to the end along the central axis of symmetry.

The length of the left fore flipper was measured from the base of the forearm protruding from the body bag along the outer edge to the tip of the claw of the first toe.

The length of the left hind flipper was measured from the base of the part of the tibia protruding from the body bag along the outer edge to the tip of the first toe.

Chest circumference – was measured under the front flippers of the animal. Hair length was measured on the scruff, from its base to the tip.

The animal was weighed. Additionally, the condition of the Caspian seal hair cover was assessed: coat color, presence of non-molting areas (for studies during the molting period). The presence of skin lesions and any other possible abnormalities was recorded. The animal was photographed. The records included the animal number, date, time and place of the study (GPS coordinates), species of the animal, sex and results of morphometric measurements. If necessary, photographs were taken: general view from above, from the left and right sides (seal lying on its belly); general view from above (seal lying on its back) for a dead animal; head; front left and rear left flipper; tail; skin and hair lesions and other abnormalities.

Measurements of the seal head were made using a caliper. Depending on the objectives of the study, the list of measurements may include: total, or condylobasal length of the skull (from the lowest point of the nostril to the junction of the skull with the cervical spine); length of the cerebral portion of the skull (from the lateral corner of the eye to the junction of the skull with the cervical spine); width of the rostrum at the upper point of the nostril; width of the cerebral portion of the skull at the medial corner of the eye; width of the cerebral part of the skull at the slit-shaped opening of the external auditory canal; length of the external slit-shaped opening of the left nostril; distance from the lateral to the medial corner of the eye; distance from the medial corner of the eye to the auditory opening; length of the slit-shaped opening of the external auditory canal.

When examining the claws on the fore flipper, attention was paid to the presence of the embryonic cone (Figure 3.3.4). Full segments were counted on the claw where the embryonic cone was not emarginated or where part of it was present and the segments were most clearly visible. Segments were also counted on the claws of hind flippers if they were clearly visible.

1 – embryonic cone; 2 - complete segments

Figure 3.3.4 Anterior Flipper Claw Studies

On the left fore and hind flipper, the length and width of the claw on the first toe were measured with a caliper. Length - from the tip to the base (up to the skin fold); width - at the base of the claw (at the skin fold).

3.3.4. Collection of Hair and Vibrissae for Toxicological Studies

Samples of wool and vibrissae were taken to determine their mercury (Hg) content and to assess the degree of mercury pollution in the Caspian Sea waters.

List of necessary equipment for bio-sampling: zip-lock bag 7 x 10 cm (80 μm), or zip-lock bag 6 x 8 cm (40 μm); surgical scissors, scalpel; general purpose tweezers; gauze wipes for instrument treatment; container for bagged samples; waterproof labels; waterproof marker; antiseptics and disinfectants - ethyl alcohol (70-80 % vol), hydrogen peroxide solution (3%). Personal protective equipment (PPE) is also required: disposable medical masks, disposable medical latex gloves.

Method of bio-sampling. The animal's wool (from the scruff of the neck, weighing about 1 g) was clipped with scissors or scalpel and placed in a bag. Vibrissae were cut out with scissors: in a live animal 1 piece above the eyes, in a fallen animal 2-3 pieces on the upper lip. Biomaterial was collected from a live seal when it was physically immobilized.

Labeling of individual packages. The packages were labeled. The label indicated: animal number; animal species (*Pusa caspica*); type of biomaterial - wool (Hg), vibrissae (Hg), date in the format DD.MM.YY.

Information reflected in the field jlog. The field logbook included animal number, date, time, place of sample collection (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, age (baby seal, sivar, yearling, immature specimen, adult specimenl), morphometric data, coat coloration; sample contents; number of samples, marking on individual label, brief description of individual package, comments.

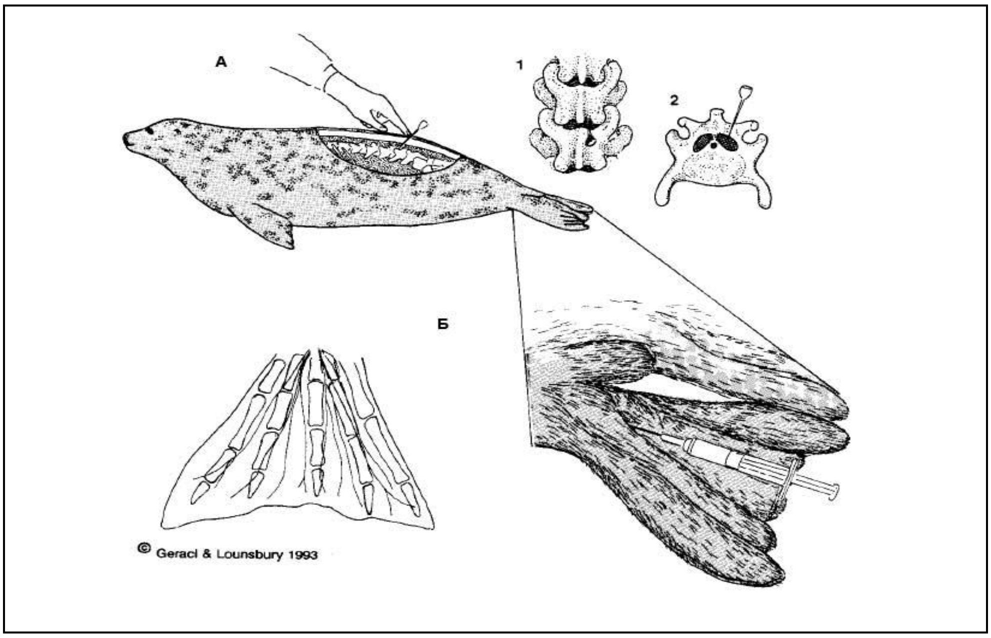
Storage and transportation. Samples were stored and transported frozen (strictly below 0°C). For short-term transportation, a thermo-bag or thermo-container with frozen cold accumulators was used.

3.3.5. Blood Sampling for Toxicological Studies

Blood was collected from a live Caspian seal for in vitro analysis of persistent organic pollutants (POPs) and toxic metals.

List of necessary equipment for bio-sampling. Sterile 21G x 3/4" (0.8 mm x 2.0 cm) butterfly needles with luer adapter and holder, sterile 20G x 1 1/2" (0.9 mm x 4 cm) needles with luer adapter, vacuum tube holders, syringes (10 ml); medical tourniquet for venous blood collection are required for blood collection from the flipper. For blood collection from the epidural venous sinus of the spinal canal it is necessary to have: spinal needles of 18G×75 mm and 18G×90 mm with a luer-adapter (chosen depending on the fatness of the animal); vacuum tube holders. In addition, sterile vacuum tubes with anticoagulant K3 EDTA for hematology (6 ml volume); antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); gauze napkins; waterproof marker; waterproof labels; small bags (10 x 15 cm) made of dense polyethylene (80 microns) with zip-lock (for storing all tubes); PPE (disposable medical masks, disposable medical latex gloves).

Method of bio-sampling. Biomaterial sampling was performed during physical immobilization of the animal. Blood samples were taken from the interphalangeal veins of the hind flipper of the Caspian seal (Figure 3.3.5).



A –blood sampling from the epidural venous sinus of the spinal canal; B - blood sampling from the hind flipper of a seal [Geraci, Lounsbury, 1998].

Figure 3.3.5 Blood Collection Technique

The needle insertion area was preliminarily disinfected with ethanol. When drawing blood from the epidural venous sinus of the spinal canal, the needle was inserted between the spinous processes (1) of the lumbar vertebrae into the epidural vein (2), which lies above the spinal cord. To draw blood from the posterior flipper, the needle was inserted into the vein lying between the second and third toe (just above the interfinger webbing). Given that 10 mL of blood plasma is needed for POP analysis and 15 mL of whole blood is needed for metal analysis, 45 mL of blood is drawn.

Individual package labeling. Blood tubes were labeled using a waterproof marker. Due to limited space, a short code was used for labeling: animal number; animal species - PC (*Pusa caspica*); biomaterial type - BPOPM (blood for POPs and metals); date of blood collection in the format DD.MM.YY.

Information in the field logbook. The field logbook included animal number, date, time, place of sample collection (GPS-coordinates); information about the animal, which is necessary for further studues: animal species, sex, age morphometric data, sample contents; number of samples, marking on individual label, brief description of individual package, comments.

Storage and transportation. Blood samples were stored and transported in the field at -15 to -30°C. For short-term transportation, a thermo-bag or thermo-container with frozen cold accumulators was used.

3.3.6. Collection of Hair and Vibrissae for Hormonal Studies

Samples of hair and vibrissae were taken from Caspian seal to determine stress hormones and sex hormones (cortisol, testosterone and progesterone) contained in them. Based on the data obtained, the reproductive condition of the animal and the stress level of certain speciments are assessed.

List of necessary equipment for bio-sampling: surgical scissors, scalpel; general purpose medical anatomical forceps; small bags (7 x 10 cm) of dense polyethylene (80 µm) with zip-lock (for individual hair samples); polyethylene bag with a capacity of 1 liter for freezing to - 30°C with zip-lock and runner (for storing all hair samples); labels; waterproof marker; antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); 50-100 ml container for rinsing tweezers in hydrogen peroxide; gauze wipes; PPE (disposable medical masks, disposable medical latex gloves).

Method of bio-sampling. Biomaterial selection was performed when the animal was physically immobilized. Hair, vibrissae were fixed with tweezers and clipped with scissors at the base of the rod. Hair was taken from the croup of the animal. In a live seal, 1 vibrissae above the eyes was cut off, in a fallen seal - 2-3 vibrissae on the upper lip. A sample of each type of material weighing 0.1-0.5 g was required for the study and was placed in an individual zip-lock bag. After sampling, scissors and forceps were rinsed thoroughly in hydrogen peroxide solution.

Labeling of individual packages. Bags with hair and vibrissae samples were labeled using a paper label and an alcohol-resistant marker. The label indicated: animal number; animal species - *Pusa caspica*; type and purpose of the biomaterial - hair (hormones) or vibrissae (hormones); date of sampling in the format DD.MM.YY.

Information in the field logbook. The field logbook included the number of the animal, date, time, place of sample collection (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, age (baby seal, sivar, yearling, immature specimen, adult specimen), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

Storage and transportation. Hair and vibrissae samples were stored and transported in the field at -15 to -30°C. A thermo-bag or thermo-container with frozen cold accumulators was used for short-term transportation.

3.3.7. Tissue Sampling for Genetic Studies

Tissue samples were collected for subsequent isolation of total DNA of a specimen for the purpose of analyzing certain genetic markers used when assessing population structure, trends in its change, population well-being, etc.

The main type of tissue collected for these purposes is whole blood. Venous blood sampling is one of the least traumatic methods for a living animal, allowing for sufficient numbers of live cells to analyze multiple DNA markers. Preserving whole blood enables to minimize the concentration of free hemoglobin and iron ions (which inhibit the polymerase chain reaction) prior to analysis.

In case of impossibility to collect a sufficient amount of whole blood for genetic analysis, the blood formed elements remaining after plasma extraction from samples collected for serologic studies are preserved, and hair with hair follicles is collected from the animal.

3.3.7.1. Blood Sampling

List of necessary equipment and materials: 2-10 ml sterile vacuum tubes for hematology studies with K2 EDTA or K3 EDTA anticoagulant; sterile 21G x 3/4" (0.8 mm x 2.0 cm) butterfly needles with luer adapter and holder, sterile 20G x 1 1/2" (0.9 mm x 4 cm) needles with luer adapter, vacuum tube holders; vacuum tube rack; antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); gauze napkins; labels; alcohol-resistant marker; PPE (disposable medical masks, disposable medical latex gloves).

Method of bio-sampling. Biomaterial selection was performed during physical immobilization of the animal. Venous blood was collected as a sample by puncturing a vein on the hind flipper of the seal. The vein puncture area was preliminarily disinfected with ethanol. The work was carried out with disposable medical gloves.

Amount of material collected. The volume of blood collected from one animal should be at least 1 ml, preferably 2-4 ml.

Labeling of individual packaging. The tube with blood was labeled - the number of the animal was indicated; type of animal - PC (*Pusa caspica*); type of biomaterial - BG (blood for genetic studues); date of blood collection in the format DD.MM.YY.

Storage and transportation. If no more than 5 days elapsed between the time of blood collection and delivery of the tube to the laboratory, it is acceptable to store tubes with collected blood both at 15 - -30°C and at +2 - +4°C. For transportation we used a thermo-bag or thermo-container with cold accumulators, the temperature of which corresponds to the temperature of tubes storage.

3.3.7.2. Tissue Sampling

Tissue samples (muscle or skin) were taken from a dead Caspian seal for genetic studies.

List of instruments and consumables for bioassay collection: sectioning knife; scalpel; surgical scissors; general purpose medical anatomical forceps; aluminum foil; small bags (10 x 15 cm) of dense polyethylene (80 μm) with zip-lock (for individual tissue and organ samples) or small plastic containers with airtight lid; 1 liter polyethylene bag for freezing down to - 30 °C with zip-lock and slider (for storage of all samples); waterproof labels; waterproof marker; antiseptics and disinfectants: Ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); gauze napkins; PPE (disposable medical masks, disposable medical latex gloves).

Method of bio-sampling. The animal autopsy was performed [Pugliares et al., 2007]. Tissue sampling was performed in the absence of visible signs of decomposition. Samples were placed in a test tube filled with ethyl alcohol. The sample was completely covered with ethyl alcohol.

Individual package labeling. The tubes with the biomaterial sample were labeled using a waterproof label and a waterproof marker. The label indicated: animal number; animal species - Pusa caspica; date of sample collection in the format DD.MM.YY.

Information in the field logbook. The field log included animal number, date, time, sampling location (GPS-coordinates); information about the animal that is necessary for further studies: animal species, sex, age morphometric data, sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

thermo-container with frozen cold accumulators was used for short-term transportation.

Storage and transportation. Biomaterial samples were stored and transported in the field at minus 15 - minus 30 ºC. A thermal bag or thermal container with frozen cold storage batteries was used for short-term transportation.

3.3.8. Blood Sampling and Obtaining Plasma for Serological Studies, and Blood Smear

Blood sampling with subsequent plasma extraction from it was performed to assess in laboratory conditions the parameters of innate immunity of Caspian seal and identification of antibodies to various pathogens: herpes simplex virus, parvovirus, carnivore plague virus and pseudorabies, to trichinella, toxoplasma, chlamydia, brucella, toxoplasma, mycoplasma, candida, etc.

List of required equipment for bioassay collection: blood collection from the flipper will require: sterile 21G x 3/4" (0.8 mm x 2.0 cm) butterfly needles with luer adapter and holder, sterile 20G x 1 1/2" (0.9 mm x 4 cm) needles with luer adapter, vacuum tube holders, syringes (10 mL). To draw blood from the epidural venous sinus of the spinal canal, 18G×75 mm and 18G×90 mm spinal needles with a luer adapter (selected according to the fatness of the animal), vacuum tube holders; sterile vacuum tubes with K3 EDTA anticoagulant for hematology (6 ml volume); are needed. Also needed: Cryo- and microtube rack, vacuum tube rack; medical laboratory centrifuge; automatic pipette and sterile disposable tips for blood plasma sampling; sterile cryopreservable sealed polypropylene tubes with screw cap (2 ml volume) for plasma/serum storage; sterile pipettes for dosage of blood and blood formers into Eppendorf tubes; sterile Eppendorf tubes (1.5-2.0 ml); antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); gauze napkins; waterproof labels; waterproof marker; PPE (disposable medical masks, disposable medical latex gloves).

Method of bio-sampling. Biomaterial selection was performed during physical immobilization of the animal. Blood samples were taken from the epidural venous sinus of the spinal canal or the hind flipper of the Caspian seal. The area of vein puncture was preliminarily disinfected with ethanol. Blood samples were centrifuged for 15 min at 3000 rpm. The obtained serum was transferred into a sterile cryoprotectable hermetically sealed polypropylene tube with a screw cap. Remaining after centrifugation, the blood formers can be used for a number of studies (toxicological, genetic, etc.).

For a blood smear, 1 μl of blood is taken with a clean spout and transferred to a sterile slide. A second sterile slide is placed at an angle of 45° relative to the first slide. A drop of blood is smeared along the length of the slide with a quick movement. The resulting smear is fixed with methanol. Storage - at room temperature in a swab container.

Labeling of the individual package. The blood tube was labeled using a waterproof marker. Due to limited space, a short code was used for labeling: animal number; animal species - PC (*Pusa caspica*); type of biomaterial - BS (blood for serological studies); date of blood collection in the format DD.MM.YY.

Blood plasma tubes were labeled using a water-resistant marker. Due to limited space, a short code was used for labeling: animal number 00; animal species - MS (Pusa caspica); biomaterial type - PS (plasma for serological studies); date of blood collection in the format DD.MM.YY.

After centrifugation, the tubes with the blood form elements were labeled with water-resistant labels and a water-resistant marker. Due to limited space, a short code was used for labeling: animal number 00; animal species - PC (*Pusa caspica*); type of biomaterial - BFE (blood form elements); date of blood collection in the format DD.MM.YY.

Blood form elements are used for toxicological, genetic and other studies.

Information in the field logbook. The field logbook included animal number, date, time, place of sample collection (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, etc.; sample contents; number of samples, marking on individual label, brief description of individual package, comments.

Storage and transportation. Samples of plasma and blood constituents were stored and transported in the field at 15 - -30°C. For short-term transportation, a thermo-bag or thermo-container with frozen cold accumulators was used.

3.3.9. Taking Swabs for Virological Studies

Conjunctival (eye) swabs from live seals were collected to identify pathogens of multisystem viral infections of animals (adenoviruses, herpesviruses, caliciviruses, coronaviruses, lyssaviruses, orthomyxoviruses, paramyxoviruses) and a number of other pathogens.

Nasal swabs from live seals were collected to identify pathogens of respiratory and multisystem viral infections of animals transmitted by airborne droplets; orthomyxoviruses (influenza infections); coronaviruses (seal coronavirus, dog coronavirus); morbilliviruses (including dog plague and seal plague); herpesviruses (alpha- and gamma-herpesviruses of seal serotypes 1-7); lyssaviruses (including rabies) and a number of other pathogens in the animal organism.

Mouth swabs from live seals were collected to identify pathogens of respiratory, gastrointestinal and multisystem viral infections of animals (adenoviruses, herpesviruses, caliciviruses, coronaviruses, lissaviruses, orthomyxoviruses, paramyxoviruses) and a number of other pathogens.

Urogenital swabs from live seals were collected to identify pathogens of multisystem viral infections of animals (adenoviruses, hepadnoviruses, herpesviruses, caliciviruses, papillomaviruses) and a number of other pathogens.

Rectal swabs from live seals were collected to identify pathogens of gastrointestinal and multisystem viral infections of animals (adenoviruses, hepadnoviruses, caliciviruses, papillomaviruses, parvoviruses) and a number of other pathogens.

List of necessary materials and equipment for bioassay collection: sterile, cryopresistant, leak-proof polypropylene tubes with screw cap (1.8-2 ml volume); virus-transport medium, e.g.: transport medium 199 containing antibiotic complex (penicillin 2000 units/mL, streptomycin 2 mg/mL, gentamicin 50 µg/mL, nystatin 50 units/mL) and bovine serum albumin at a final concentration of 0.5% (0.8 ml of the mixture is added to each tube); nylon (dacron or viscous aplicates). /ml) and bovine serum albumin at a final concentration of 0.5% (0.8 ml of the mixture is added to each tube; nylon (dacron) or viscose applicators with plastic rod; scissors; cryoprobe rack; a self-contained portable field printer for label printing, e.g. BRADY type BMP21, a cartridge with liquid nitrogen stable self-adhesive nylon tape (12.7 mm wide) and a multicolored permanent marker; antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); spray bottle for disinfectants; paper napkins; personal protective equipment: disposable medical masks, disposable medical latex gloves; Dewar vessel with liquid nitrogen or portable freezer (- 80°C).

Method of bio-sampling

Ocular swabs. Biomaterial was collected when the animal was physically immobilized. To collect lacrimal fluid and epithelial cells, it is necessary to carefully pass the cotton tip of the applicator along the conjunctiva at the lower edge of the eye slit, paying attention to the lateral corner of the eye. Next, place the applicator in a cryotube with transport medium and cut the rod. Two swabs should be collected from one animal. Place each applicator in a separate tube.

Nasal swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully insert the tip of the applicator into the animal's nostril and make several rotational movements so that besides the nasal discharge, the cells of the mucous membrane epithelium fall on it (the applicator is inserted into each nostril; the depth of insertion is up to 4 cm). Next, place the applicator in a cryotube with virus-transporting medium and cut off the rod. Two f swabs should be taken from one animal. Place each applicator in a separate tube.

Mouth swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully pass the absorbent tip of the applicator along the inner sides of the cheeks and oropharynx, paying attention to the tonsillar secretion. Next, place the applicator in a cryotube with virus-transporting medium and cut off the rod. Two swabs should be sampled from one animal. Place each applicator in a separate tube.

Urogenital swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully pass the absorbent tip of the applicator along the inner sides of the female genital tract or male prepuce, paying attention to the secretion. Next, place the applicator in a cryotube with virus-transporting medium and cut off the rod. Two swabs should be sampled from one animal. Place each applicator in a separate tube.

Rectal swabs. Biomaterial sampling was performed when the animal was physically immobilized. To collect rectal swabs and fecal samples, the applicator should be carefully inserted into the anus (insertion depth up to 5-6 cm) and passed along the inner sides of the intestine. Next, place the applicator in a cryotube with virus transport medium and cut the rod. Two swabs should be sampled from one animal. Place each applicator in a separate tube [Manual of standards, 2019].

Labeling of individual packaging. Test tubes with samples were labeled using self-adhesive nylon tape, overlapped. An animal code consisting of the Latin two-letter designation of the place of material collection, the year of collection at the specified point, and the two-digit serial number of the animal was used for labeling. For example: KU-22/01 (KU - Kulaly, 2022, 01 - animal number). The type of biomaterial is indicated - ocular swab (eye swab), nasal swab (nasal swab), buccal swab (oral swab), genital swab (urogenital tract swab), rectal swab (rectal swab); animal species - *Pusa caspica*, date of material collection.

Field logbook information. The field logbook included animal number, date, time, sampling location (GPS-coordinates); information about the animal that is necessary for further studies: animal species, sex, age (belek, sivar, yearling, immature, adult), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

Storage and transportation. Samples were stored in liquid nitrogen (-196ºC) during fieldwork and transported in a special Dewar vessel recommended by IATA for air transportation. Swabs were allowed to be stored at 2 to 8°C for 1-2 days; at -18°C for 2-3 weeks. At -80°C or in liquid nitrogen samples can be stored for a long time. Only single freezing and thawing of biomaterial is possible.

3.3.10. Taking of Swabs for Molecular andVirological Studies

Conjunctival (eye) swabs from live seals were collected for mass parallel sequencing of virome in NGS (Next Generation Sequencing) and detection of nucleic acids of pathogens of multisystem viral infections of animals (adenoviruses, herpesviruses, caliciviruses, coronaviruses, lyssaviruses, orthomyxoviruses, paramyxoviruses) and some other pathogens.

Nasal swabs from live seals were collected for mass parallel virome sequencing by NGS and nucleic acid detection of pathogens of respiratory and multisystem viral infections of animals transmitted by airborne respiratory infection in polymerase chain reaction (PCR); orthomyxoviruses (influenza infections); coronaviruses (coronavirus of seals, coronavirus of dogs); morbilliviruses (including plague of dogs and plague of seals); herpesviruses (alpha- and gamma-herpesviruses of seals of serotypes 1-7); lyssaviruses (including rabies) and a number of other pathogens in the animal organism.

Mouth swabs from live seals were collected for mass parallel virome sequencing and nucleic acid detection of respiratory, gastrointestinal and multisystem animal viral pathogens (adenoviruses, herpesviruses, caliciviruses, coronaviruses, lyssaviruses, orthomyxoviruses, paramyxoviruses) and several other pathogens.

Urogenital swabs from live seals were collected for mass parallel virome sequencing and nucleic acid detection of pathogens of multisystem viral infections of animals (adenoviruses, hepadnoviruses, herpesviruses, caliciviruses, papilomaviruses) and a number of other pathogens.

Rectal swabs from live seals were collected for mass parallel virome sequencing and nucleic acid detection of pathogens of gastrointestinal and multisystem viral infections of animals (adenoviruses, hepadnoviruses, caliciviruses, papilomaviruses, parvoviruses) and a number of other pathogens.

List of items needed for bioassay collection: sterile polypropylene tubes with screw cap (volume 1. 8-2 ml; DNA/RNA Shield reagent to stabilize nucleic acids in any biological sample, maintains genetic integrity and expression profiles of samples at ambient temperature and completely inactivates infectious agents (viruses, bacteria, fungi and parasites), 0.8 ml of reagent should be added to each tube; sterile nylon (dacron) or viscose applicators with plastic stem; scissors; microtube rack; self-contained portable field label printer, e.g. BRADY type BMP21; cartridge with self-adhesive nylon tape (12.7 mm wide) and multicolored permanent marker for labeling. Antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); spray bottle for disinfectants; paper napkins; personal protective equipment: disposable medical masks, disposable medical latex gloves.

Method of bio-sampling

Ocular swabs. Biomaterial was collected when the animal was physically immobilized. To collect lacrimal fluid and epithelial cells, it is necessary to gently run the cotton tip of the applicator along the conjunctiva at the lower edge of the eye slit, paying attention to the lateral corner of the eye. Next, place the applicator in a tube of DNA/RNA Shield reagent and cut the rod.

Nasal swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully insert the tip of the applicator into the animal's nostril and make several rotational movements so that besides the nasal discharge, the mucous membrane epithelial cells fall on it (the applicator is inserted into each nostril; the depth of insertion is up to 4 cm). Next, place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Oral swabs. Biomaterial sampling was performed while the animal was physically immobilized. It is necessary to gently run the absorbent tip of the applicator along the inner sides of the cheeks and oropharynx, paying attention to the tonsillar secretion. Next, place the applicator in a tube of DNA/RNA Shield reagent and cut off the rod.

Urogenital swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully pass the absorbent tip of the applicator along the inner sides of the mother's genital tract or father's prepuce, paying attention to the secretion. Next, place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Rectal swabs. Biomaterial sampling was performed when the animal was physically immobilized. To collect rectal swabs and fecal samples, the applicator should be carefully inserted into the anal opening of the animal (insertion depth up to 5-6 cm) and run along the inner sides of the intestine. Then place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Individual package labeling. Sample tubes were labeled using self-adhesive overlapping nylon tape. An animal code consisting of the Latin two-letter designation of the place of material collection, the year of collection at the specified point, and the two-digit serial number of the animal was used for labeling. For example: KU-22/01 (KU - Kulaly, 2022, 01 - animal number). The type of biomaterial was indicated - ocular NA, nasal NA, buccal NA (oral NA), genital NA (urogenital NA), rectal NA; animal species - *Pusa caspica*, date of material collection.

Information in the field logbook. The field logbook included the animal number, date, time, sampling location (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, age (baby seal, sivar, yearling, immature specimen, adult specimen), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

Storage and transportation. For conjunctival, nasal, oral, urogenital and rectal swabs, storage in DNA/RNA Shield reagent is allowed at 35-40°C for up to 7 days; at 4-25°C for up to 30 days. At temperatures of -20°C and below, samples may be stored for extended periods of time.

3.3.11. Taking of Swabs for Molecular and Bacterial Studies

Conjunctival (eye) swabs from live seals were collected for mass parallel sequencing of the 16S mRNA gene of the animal eye mucosal microbiome and bacterial pathogens of corneal infectious diseases in NGS (Next Generation Sequencing): pseudomonosis, moraxelosis, klebseliasis, leptospirosis.

Nasal swabs from live seals were collected for massively parallel sequencing of the 16S mRNA gene of the nasal cavity microbiome and bacterial pathogens of animal respiratory diseases in NGS: aeromonosis, bordotheliosis, staphylococcosis, streptococcosis, mycoplasmosis, leptospirosis.

Mouth swabs from live seals were collected for mass parallel sequencing of the 16S mRNA gene of the oral microbiome and pathogens of multisystem bacterial infections of animals in NGS (diplococcosis, bordotheliosis, staphylococcosis, streptococcosis, leptospirosis, listeriosis, mycoplasmosis, pasteurellosis) and a number of other pathogens.

Urogenital swabs from live seals were collected for mass parallel sequencing of the 16S mRNA gene of the genital tract microbiome and bacterial pathogens of multisystem animal infections in NGS (aeromonosis, brucellosis, leptospirosis, edwardsiellosis, pasteurellosis and a number of other pathogens).

Rectal swabs from live seals were collected for massively parallel sequencing of the 16S mRNA gene of the gastrointestinal microbiome and bacterial pathogens of animal intestinal infections by NGS (salmonellosis, clostridiosis, campylobacteriosis and several other pathogens).

List of items needed for bioassay collection: sterile polypropylene tubes with screw cap (1.8-2 ml volume); DNA/RNA Shield reagent - to stabilize nucleic acids in any biological sample, maintains genetic integrity and expression profiles of samples at ambient temperature and completely inactivates infectious agents (viruses, bacteria, fungi and parasites), 0.8 ml of reagent should be added to each tube; sterile nylon (dacron) or viscose applicators with plastic stem; scissors; microtube rack; self-contained portable field label printer, e.g. BRADY type BMP21; cartridge with self-adhesive nylon tape (12.7 mm wide) and multicolored permanent marker for labeling; antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); spray bottle for disinfectants; paper napkins; personal protective equipment: disposable medical masks, disposable medical latex gloves.

Method of bio-sampling

Ocular swabs. Biomaterial was collected when the animal was physically immobilized. To collect lacrimal fluid and epithelial cells, it is necessary to gently run the cotton tip of the applicator along the conjunctiva at the lower edge of the eye slit, paying attention to the lateral corner of the eye. Next, place the applicator in a tube of DNA/RNA Shield reagent and cut the rod.

Nasal swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully insert the tip of the applicator into the animal''s nostril and make several rotational movements so that besides the nasal discharge, the mucous membrane epithelial cells fall on it (the applicator is inserted into each nostril; the depth of insertion is up to 4 cm). Next, place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Oral swabs. Biomaterial sampling was performed while the animal was physically immobilized. It is necessary to gently run the absorbent tip of the applicator along the inner sides of the cheeks and oropharynx, paying attention to the tonsillar secretion. Next, place the applicator in a tube of DNA/RNA Shield reagent and cut off the rod.

Urogenital swabs. Biomaterial sampling was performed when the animal was physically immobilized. It is necessary to carefully pass the absorbent tip of the applicator along the inner sides of the genital tract of females or prepuce of males, paying attention to the secretion. Next, place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Rectal swabs. Biomaterial sampling was performed when the animal was physically immobilized. To collect rectal swabs and fecal samples, the applicator should be carefully inserted into the anal opening of the animal (water depth up to 5-6 cm) and run along the inner sides of the intestine. Then place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Individual package labeling. Sample tubes were labeled using self-adhesive overlapping nylon tape. An animal code consisting of the Latin two-letter designation of the place of material collection, the year of collection at the specified point, and the two-digit serial number of the animal was used for labeling. For example: KU-22/01 (KU - Kulaly, 2022, 01 - animal number). The type of biomaterial is indicated - ocular NA, nasal NA, buccal NA (oral NA), genital NA (urogenital NA), rectal NA; animal species - *Pusa caspica*, date of material collection.

Information in the field logbook. The field logbook included the animal number, date, time, sampling location (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, age (baby seal, sivar, yearling, immature specimen, adult specimen), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

Storage and transportation. For conjunctival, nasal, oral, urogenital and rectal swabs or swabs, storage in DNA/RNA Shield reagent is allowed at 35-40°C for up to 7 days; at 4-25°C for up to 30 days. At temperatures of -20°C and below, samples may be stored for extended periods of time.

3.3.12. Taking of Rectal Swabs or Fresh Fecal Samples for Molecular Parasitological Studies

Rectal swabs from live seals were collected for mass parallel sequencing of 16S mtDNA, 12S mtDNA, 28S rDNA genes of animal helminth fauna in NGS (Next Generation Sequencing).

List of necessary materials for bioassay collection: sterile polypropylene tubes with screw cap (volume 1. 8-2 ml); DNA/RNA Shield reagent - to stabilize nucleic acids in any biological sample, maintains genetic integrity and expression profiles of samples at ambient temperature and completely inactivates infectious agents (viruses, bacteria, fungi and parasites), 0.8 ml of reagent should be added to each tube; sterile nylon (dacron) or viscose applicators with plastic stem; scissors; microtube rack; self-contained portable field label printer, e.g. BRADY type BMP21; cartridge with self-adhesive nylon tape (12.7 mm wide) and multicolored permanent marker for labeling; antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); spray bottle for disinfectants; paper napkins; personal protective equipment: disposable medical masks, disposable medical latex gloves.

Method of bio-sampling

Rectal swabs. Biomaterial sampling was performed when the animal was physically immobilized. To collect rectal swabs and fecal samples, the applicator should be carefully inserted into the anal opening of the animal (insertion depth up to 5-6 cm) and run along the inner sides of the intestine. Next, place the applicator in a tube with DNA/RNA Shield reagent and cut off the rod.

Fecal samples. To collect fecal samples, insert the applicator into the thickness of the feces and swipe several times. Then place the applicator in a tube with DNA/RNA Shield and cut off the rod.

Labeling of individual packaging. Sample tubes were labeled using self-adhesive overlapping nylon tape. An animal code consisting of the Latin two-letter designation of the place of material collection, the year of collection at the specified point, and the two-digit serial number of the animal was used for labeling. For example: KU-23/01 (KU - Kulaly, 2023, 01 - animal number). Type of biomaterial - rectal NA, animal species - *Pusa caspica*, date of material collection are indicated.

Information in the field logbook. The field logbook outlined the number of the animal, date, time, place of sample collection (GPS-coordinates); information about the animal, which is necessary for further studues: animal species, sex, age (baby seal, sivar, yearling, immature specimen, adult specimen), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual label, and a brief description of the sample.

Storage and transportation. Rectal swabs and fecal samples may be stored in DNA/RNA Shield reagent at 35-40°C for up to 7 days; at 4-25°C for up to 30 days. At temperatures of -20°C and below, samples can be stored for a longer period of time.

3.3.13. Taking of Swabs for Microbiological Studies

Nasal, anal and vaginal swabs are collected to identify pathogenic microorganisms that pose a threat to the well-being of the species or are potentially hazardous to it.

List of equipment, tools and consumables for bio-sampling: polymer tubes with a filler (probe with a viscose tip); protective equipment: disposable medical masks made of non-woven material and disposable medical latex gloves; antiseptics and disinfectants: ethyl alcohol (96% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); lacquer marker for marking samples.

Bio-sampling method: nasal, anal and vaginal swabs were collected by inserting the entire tip of the probe inside the nasal passages, anus and vagina of the animal, respectively. The probe was rotated in two to four circular motions so that, in addition to the secretion, the mucosal epithelial cells fell on it. Each probe was placed in a separate sterile tube. Nine swabs were taken from one animal: three nasal, three anal and three vaginal. Swab tubes were labeled using a lacquer marker. Information on sample collection was recorded in the field protocol.

Labeling of individual packages. Test tubes with samples are labeled using self-adhesive nylon tape applied overlapping. An animal code consisting of the Latin two-letter designation of the place of material collection, the year of collection at the specified point, and the two-digit serial number of the animal is used for labeling. For example: PR.2019-09 (PR - Prorva, 2019, 09 - animal number). Type of biomaterial - N (nasal), A (anal), V (vaginal); animal species - PC (*Pusa caspica*), date of material collection are indicated.

Field logbook information. The field logbook specifies animal number, date, time, sampling location (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, age (bby seal, sivar, yearling, immature specimen, adult specimen), morphometric data, coat coloration; sample contents; number of samples, marking on individual label, brief description of individual package, comments.

Storage and transportation. Selected swabs are stored and transported in the field at a temperature of minus 15 - minus 30 ºC. For short-term transportation, a thermo-bag or thermo-container with frozen cold accumulators is used.

3.3.14. Tissue Collection for Virological Studies

Brain, lung, heart, liver, kidney and lymph node tissues from fallen seals were collected for identification, isolation and molecular diagnosis of animal viral infections; orthomyxoviruses (influenza infections); coronaviruses (seal coronavirus, dog coronavirus); morbilliviruses (including dog plague and seal plague); herpesviruses (alpha- and gamma-herpesviruses of seal serotypes 1-7); lyssaviruses (including rabies) and a number of other animal pathogens.

List of items required for bioassay collection: sterile polypropylene tubes with screw cap (volume 1. 8-2 ml; virus-transport medium 199 containing antibiotic complex (penicillin 2000 units/mL, streptomycin 2 mg/mL). 8-2 mL); virus-transport medium, e.g., Transport Medium 199 containing an antibiotic complex (penicillin 2000 units/mL, streptomycin 2 mg/mL, gentamicin 50 µg/mL, nystatin 50 units/mL) and bovine serum albumin at a final concentration of 0.5% (0.8 mL of the mixture should be added to each tube); DNA/RNA Shield reagent to stabilize nucleic acids in any biological sample, maintains genetic integrity and expression profiles of samples at ambient temperature and completely inactivates infectious agents (viruses, bacteria, fungi and parasites), 0.8 ml of reagent should be added to each tube; sterile nylon (dacron) or viscose applicators with a plastic rod; disinfection boiling pot; veterinary small amputation knife; resection abdominal knife; cartilaginous rib knife; anatomical intestinal straight scissors; rib scissors; blunt-tipped straight scissors; anatomical gloves; plastic-handled sheet saw; general purpose anatomical forceps; scalpel, e.g., veterinary medium abdominal scalpel and scalpel tip for detachable blades in sizes 21-25; microtube rack; self-contained portable field printer, e.g., BRADY type BMP21; cartridge with self-adhesive nylon tape (12.7 mm wide) and multicolored permanent marker for marking; antiseptics and disinfectants: ethyl alcohol (70-80% vol.); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%); spray bottle for disinfectants; paper napkins; personal protective equipment: disposable medical masks, disposable medical latex gloves.

Bio-sampling method. The autopsy of the animal was performed [Pugliares et al., 2007].

The brain should be examined by removing the upper part of the skull with a saw. Brain sections of 1 cm^3 each should be taken under aseptic conditions and placed in a cryotube with virus transport medium and tubes with DNA/RNA Shield reagent.

Open the chest by removing the lower part of the wall with bone scissors. Under aseptic conditions, take a 1 cm3 slice of lung, spleen and bronchial lymph node tissue and place it in a cryoprobe with virus-transporting medium and tubes with DNA/RNA Shield reagent.

Uncover the chest by removing the lower part of the wall with bone scissors. Under aseptic conditions, take a 1 cm3 slice of lung, spleen and bronchial lymph node tissue and place it in a cryotube with virus-transport medium and tubes with DNA/RNA Shield reagent.

Labeling of individual package. Sample tubes were labeled using self-adhesive overlapping nylon tape. For labeling, we used the animal code consisting of the Latin two-letter designation of the place of material collection, the year of collection at the specified point, and the two-digit serial number of the animal. For example: KU-22/01 (KU - Kulaly, 2022, 01 - animal number). Specify the type of biomaterial - brain, lung, spleen, bronchi lymph node, animal species - *Pusa caspica*, date of material collection.

Field logbook information. The field logbook included animal number, date, time, sampling location (GPS-coordinates); information about the animal, which is necessary for further studies: animal species, sex, age (baby seal, sivar, yearling, immature specimen, adult), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

Storage and transportation

Samples of brain, lung, heart, liver, kidney and lymph node tissues from fallen seals were stored during field work in liquid nitrogen (-196 ºC) and transported in a special Dewar vessel recommended by IATA for air transportation. It is allowed to store samples placed in virus-transport medium at 2 to 8°C for 1-2 days; at -18°C for 2-3 weeks; at -80°C or in liquid nitrogen for a long time. Only single freezing and thawing of biomaterial is possible.

Samples placed in DNA/RNA Shield reagent can be stored at 35-40°C for up to 7 days; at 4-25°C for up to 30 days. At temperatures of -20°C and below, samples can be stored for a longer period of time.

3.3.15. Biological Material Collection for Morphological Studies

Samples of tissues, skin derivatives, seal organs were collected for studies by light and scanning electron microscopy methods.

The list of necessary equipment for selection and fixation of bio-samples: Sectioning knife; disposable scalpel; medical scissors; general purpose medical anatomical tweezers; plastic test tubes and small plastic containers with airtight lids; labels; waterproof marker; laboratory parafilm tape; plastic zip-lock bags; thermal bag or shipping container; antiseptics and disinfectants (ethyl alcohol (96%); chlorhexidine bigluconate solution (20%); hydrogen peroxide solution (3%)), gauze napkins; personal protective equipment (medical gown, disposable medical dressing, disposable gloves); 10% buffered (neutral) formalin solution; container for transportation of biomaterial in primary packaging; field logbook.

Method of bio-sampling. Samples of skin, claws, vibrissae, hair, stomach, large intestine, liver, kidneys were taken from a fallen animal. Hair and vibrissae samples were taken from a live animal. A 10% neutral formalin solution was used to fix the biomaterial. The ratio of bio-sample and solution was 1:10.

Hair sampling. Hair (a small bundle - 15-20 pieces) was cut with scissors or scalpel from the withers of the animal and with the help of tweezers placed in a plastic tube with a sealed lid, filled in advance with 96% ethanol.

Vibrissae collection. In a live animal, scissors were used to cut one vibrissae above the eyes, in a fallen animal - 2-3 pieces on the upper lip The specimens were placed in a sealed plastic tube or a sealed container filled in advance with 96% ethanol.

Skin sampling. The skin (together with the hair cover) was separated from the subcutaneous fatty tissue with a scalpel. Then the obtained fragment was cut with scissors into pieces of about 0.5x0.5 cm in size. Three samples prepared in this way were sufficient for the study. The obtained samples were placed in a sealed plastic tube or a sealed container with formalin.

Autopsy of the animal was performed [Pugliares et al., 2007].

Claw sampling. Five claws from the fore flipper were cut at the joint of the distal phalanx and placed in a plastic bag wit

h a zip-lock closure.

All tubes and containers with biomaterial were labeled, the neck of each tube was additionally wrapped with laboratory parafilm tape, placed in a secondary packaging with a zip-lock closure and a thermo bag or transport container.

Labeling of individual package. The label indicated: animal number; animal species (*Pusa caspica* Gmelin, 1788); type and purpose of the biomaterial (e.g., skin /morphology/ or hair /microanalysis); date of sample collection in the format DD.MM.YY.

logbook information. The field log included animal number, date, time, sampling location (GPS-coordinates); information about the animal that is necessary for further studies: animal species, sex, age, weight (baby seal, sivar, yearling, immature specimen, adult specimen), morphometric data, coat coloration; sample contents; number of samples, marking on the individual label, brief description of the individual package, comments.

Storage and transportation. Samples were stored and transported frozen. For short-term transportation a thermo-bag or thermo-container with cold accumulators was used.

3.3.16. Animals Tagging

Tag Description:

Pulsar series satellite radio tags manufactured by Es-Pass CJSC, Russia, were used for tagging (Figure 3.3.6). The radio beacon functions as part of the international satellite-based Argos positioning and data collection system, which makes it possible to determine the animal's location based on the Doppler effect (Table 3.3-2).

Figure 3.3.6 Pulsar Series Radiotag

Table 3.3-2 Pulsar Radio Beacon Specifications

Radio signal:	
Carrier frequency	401,650±MHz
Radiated signal power	700 mW
Type of signal modulation	Phase manipulation with index 1.1 radians and bit rate 400 Hz
Radiation period	1 min
Determinining of coordinates:	
Method of determining location	based on the Doppler effect of the Argos system
Operation:	
Ambient temperature	from -40 to +60^{0}C
Storage temperature	from -10 to +10^{0}C
Duration of operation	at least 12 months
Design:	
Power supply	lithium batteries
Switch	removable magnetic
Cabinet	monolithic, sealed

Antenna	pin, flexible
Weight	max. 350 g

All transmitters operate on the frequencies of the Argos satellite system (ARGOS, Advanced Research and Global Observation Satellite), a joint French-American program for space-based monitoring of the natural environment. Radio signals from the tag are detected by a satellite flying over the area. In order for the satellite to receive the signal from the transmitter, the transmitter and antenna must be completely above water. To obtain relatively accurate information about the animal's location, it is necessary for the satellite to receive a minimum of 4 messages from the transmitter: 3 to determine the location by the Doppler frequency shift effect, and the fourth to estimate the transmitter's offset relative to its own position. After receiving the data, the spacecraft transmits it to the data center.

The data received have different accuracy classes: low accuracy data have letter designations - A, B, Z and 0, higher numerical designations - from 1 to 3. The accuracy class reflects the radius of the oval in which a given point can be located and also expresses the probability of error (Table 3.3-3; see http://www.argos-system.org/).

Table 3.3-3 Accuracy Class Error

Class	Error (m)
3	<250
2	250m < < 500
1	500m < < 1500
0	>1500
A	No estimate
B	No estimate
Z	Incorrect location

Tag placement on the seal:

The Pulsar tag was attached to the animal's back between the shoulder blades [Fedak et al., 1983; Mazzaro and Dunn, 2009]. The wool in the tagging area was preliminarily cleaned of sand and dirt and degreased with medical alcohol and acetone. The transmitter surface was also degreased to maximize adhesion [Stewart et al., 1989]. After these preparations, a layer of fast-drying (five-minute) epoxy resin was applied to the wool and the tag. The surfaces were pressed against each other and cured for 15 minutes.

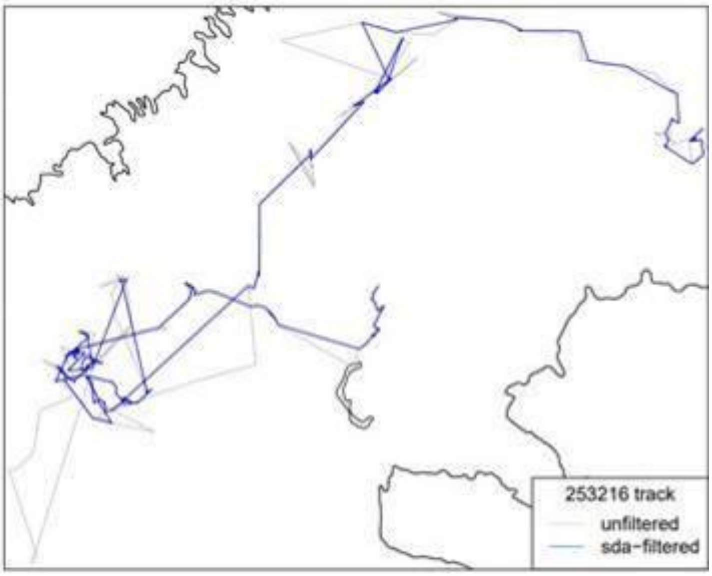
No medical drugs were used to sedate the animals. The condition of the animal was constantly monitored by specialists, preventing the seal from overheating, injuring or suffocating.

Satellite data processing:

Information on the location of animals was read from the information pool of ARGOS CLS Company via the Internet. The received data were decoded using the manufacturer's software (WC-DAP 3.0, Wildlife Computers) and pre-filtered using the Kalman Filtering algorithm on the Argos website (www.argos-system.cls.fr). The received data are “raw” and must pass through three stages of filtering before being analyzed - pre-filtering, filtering of the track itself and filtering by geographical principle.

Pre-filtering to remove repeated and erroneous messages was performed in Ms. Excel and applied to all data.

Track filtering was performed for each transmitter individually by the SDA filter (speed-distance-angle) of the argosfilter package for R [Freitas et al., 2008; R Development Core Team, 2011]. The filter takes into account the maximum movement speed, the angle between three consecutive locations, and the distance between them. The following parameters were used in the filtering: the maximum movement speed of seals was 1.5 m/s [Watanabe et al., 2004]; the maximum angle between two path segments longer than 2.5 km was 15° and 5 km was 25°. After passing this filter, all locations obtained erroneously were removed (Figure 3.3.7).



Denotations: unfiltered - track before applying the filter; sda-filtered - track after applying sda-filter

Figure 3.3.7 Schematice Map of Spatial Data Filtering on Tag Example No. 252216 using SDA-Filter

Spatial filtering by geographic principle was performed in ArcMap 10.4.1 software package. During this stage, all locations more than 1 km inland from the coastline were removed from the tracks.

The data that passed through all filters were used for further work.

Identification of key water areas:

The fixed contour method, or kernel method, [Worton, 1989] was used to determine the probability of an animal using a water area. This is a non-parametric method often used to estimate the probability of an animal occurring at each point in space. The calculations are performed using a 2-dimensional probability density function (PDF), also known as the kernel function or kernel function. This function calculates the density of point objects around each location. As a result, a set of polygons is defined, each of which has a different probability of encountering an animal, that is, the regularity of the animal's use of space is estimated. In this case, the density of point distribution is in inverse dependence on the probability of finding [Citta et al., 2012].

The maps display in the form of polygons (core areas) with a percentage score. Thus, a polygon with 90% probability is an area within which the tracked animal was almost always located, and with 10% probability - rarely, but the density of points in it is maximum, and therefore it is the most important for the animal. Traditionally, 50% and 95% polygons are used for description. Calculations were performed using the Home range and Animal movements packages for the ArcView GIS 3.2 software [Powell, 2000]. Analysis incorporated all locations obtained during the time period studied.

Calculation of activity and movement rate

Out of the resulting set of points, for each day for which data were available, one point was selected that was closest to 12:00:00:00 in terms of time with the best quality indicator (Location quality field). The selected points were connected and their length was calculated for the resulting segments, which was the distance traveled by the seal during the given time interval. This distance traveled over a certain period of time (a day, a month or the entire tracking period) was used to characterize the activity of seals. Seal movement speed (km/h) was calculated by dividing the length of the segment by the number of hours between two points. Activity and movement speed were determined for the November-December-January period only, as there was insufficient data for this analysis in February (tags did not transmit points every day).

Statistical processing of data was performed using the standard tool - Descriptive statistics in the STATISTICA 12 software.

Bathymetry Data Analysis

GebcoMaps depth maps for the Caspian Sea (http://www.gebco.net) were used to analyze bathymetry. The accuracy of the used maps is 1 m, spatial resolution is 00°00'30".

Identity analysis was performed in ArcMap 10.4.1 software package using the “"Identity” tool. This tool calculates the geometric intersection between input objects and identity objects. Attributes of the respective identity objects are attached to the input objects or parts of them that match the identity objects.

For each location, the depth of the water area in which it was located was determined. Further analysis was performed and visualized in Statistica 8 and Microsoft office software packages.

Analysis of ice conditions

For the analysis of ice conditions in the areas of animal movements, the maps of the State Scientific Center “Arctic and Antarctic Research Institute” available in the public domain (http://www.aari.ru) were used. The maps are generated once every one or two weeks (depending on the season of the year). They contain information on ice presence, ice type, ice cohesion and other features.

Such ice parameters as its type and ice cohesion were used for further analyses:

1. Ice type [Snezhinsky, 1954]:

- Nilas is the initial type of ice development. Thin, elastic ice crust, easily deflecting on waves and ripples and forming serrated layers when compressed. It has a matte surface and is up to 10 cm thick;
- Young ice - includes the concepts of “gray ice” and “gray-white ice” found in some, more detailed classifications. For our purposes, the general concept was sufficient. It is a transitional form of ice, 10 to 30 cm thick;
- One-year ice - a large group of ice, including a cover from 30 to 200 cm thick that has existed for no more than one winter.

All listed ice types exist only for one year. Perennial ice covering water for more than one consecutive year does not form in the surveyed water areas. For areas not covered by ice at all, the concept of “clear” or “open water” was used.

2. Ice cohesion is a conditional value characterizing the degree of water surface coverage by drifting ice. Its quantitative value is assessed through the ratio of the total ice area to the total area of the visible sea surface. Cohesion is measured on a ten-point scale, with 10 points corresponding to solid ice and 0 points to clear water. In some sources, the scores are converted to percentages, ranging from 0 to 100 [Ice Cohesion, 1987].

Identity analysis was performed in ArcMap 10.4.1. software package to compare animal movement data with ice data. The Identity tool attaches to each input object (location) the attributes of the respective identity objects (ice maps) with which it matches. For each ice map, only those locations that matched in terms of time interval were selected. Data on ice type, its cohesion and (if available) mobility were recorded in the attributes of each location. In case the locations fell on open water, the attributes were marked as ice-free (“clear”). Analysis and comparison of ice conditions between seasons and in different water areas was made in Microsoft excel program.

3.4. Analytical Methods

3.4.1. Toxicological Tests for Heavy Metals Content

Prior to analytical work, samples of Caspian seal hair were stored in a freezer at -10°C.

Mercury content in hair and vibrissae was measured using atomic absorption spectrometer with Zeeman correction RA 915M with pyrolytic attachment PIRO (State Register Certificate RI RF 59385-14, factory No. 2972 certificate of verification 243/195-2020). To determine mercury, a pre-weighed sample of seal hair (20-50 mg) was placed in the PIRO pyrolytic chamber for thermal decomposition with simultaneous adsorption of mercury. Mercury atoms were detected by flameless atomic absorption using a RA-915M mercury analyzer. The results of the analysis are displayed using special software RAPID.

Mercury in vibrissae was measured in a similar way.

When determining mercury in blood, 100 µl of the sample was sorbed on calcined activated carbon and then placed in a pyrolytic chamber. The remaining blood sample was lyophilized. After drying, the mercury content was measured in 10-20 mg of dry blood.

Mercury concentration in one sample of wool and blood was measured in triplicate. The number of repetitions in the analysis of vibrissae depended on the volume of incoming sample (1-6 repetitions).

DORM-2 standard samples (fish protein, NRC-CNRC, Canada) and internal standard IIWB RAS (pikeperch muscle) were used to control the accuracy of measurements (Table 3.4-1). For this purpose, the mercury content of the standard samples was measured prior to analysis. The error was 3-5%, which corresponds to the permissible error of the measurement method.

Table 3.4-1 Quality Control

Measured parameters	Analysis date	Standard sample	Concentration according to certificate, ppm	Measurement results, ppm
Mercury	1.12.2023	DORM-2	4.47±0.32	4.40±0.10
Mercury	1.12.2023	Pikeperch muscles	2.22±0.20	2.25±0.05

3.4.2. Hormonal Studies

The hormonal status of the animals was assessed using ELISA kits of Immunotech (Moscow, Russia) for measurements of cortisol, testosterone, progesterone and free triiodothyronine levels. The evaluation was performed in paired measurements with calculation of the coefficient of variation for two measured samples. If the coefficient of variation was greater than 5%, measurements were repeated. If high (outside the standard curve) hormone concentrations were detected, they were diluted with PBS and remeasured. Concentrations of progesterone and testosterone were used as a parameter to assess the activity of the sexual system of animals, the concentration of cortisol allowed to assess the level of stress of animals at a given time, the concentration of triiodothyronine (TT) characterized the intensity of metabolism in the body, to a certain extent reflecting the provision of animals with feed (the higher T3, the more the animals were provided with feed). It should be noted that the level of cortisol can also increase with feed deficiency.

Hormone extraction from hair was performed according to a previously described method [Mastromonaco et al., 2014]. Hair without hair follicles was washed for two minutes in 100% methanol (removing potential hormone residues that might have been deposited in saliva or blood) and then dried at +50°C. The dried hairs were shredded with scissors and weighed in a 15 ml tube. The weight of hair (typically 0.03-0.12 g) used for extraction was recorded to the nearest 0.0001 g (0.1 mg), and then 100% methanol was added at a rate of 10 mL of methanol per 0.1 g of hair (i.e., 5.45 mL of methanol was added per 0.0545 g of hair). The tubes were hermetically sealed and the samples were placed for 24-hour extraction in a Bio RS-24 Mini-Rotator (Biosan, South Korea). After extraction, 2 ml of solution was taken with a reusable pipette and placed in an Eppendorf tube for evaporation at +55°C. After complete evaporation of methanol, 0.4 mL of phosphate-salt buffer (PBS) was added to the tube to dissolve the hormones. All measurements were performed according to the manufacturer's instructions, if necessary (high hormone concentrations) samples were diluted with PBS at a 1:5 ratio. Similarly, extraction was performed for vibrissae, preliminarily dividing them into two-centimeter sections reflecting specific periods in the life of the animals.

Measurements were performed using commercial kits from HEMA (Moscow) for the determination of cortisol (REF K210S), testosterone (REF K209S) and progesterone (REF K207S) levels in saliva. Standards were made from the manufacturer's standards by serial dilution. The concentrations of the standards were 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 ng/mL for cortisol (kit sensitivity was 0.035 ng/mL), 0. testosterone, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 ng/mL (sensitivity - 0.005 ng/mL), progesterone - 0, 0.125, 0.25, 0.5, 1, 2, 4, 8 ng/mL (sensitivity - 0.016 ng/mL). All measurements were performed according to the manufacturer's protocols. The obtained concentration in solution X was used to calculate the concentration of hormone in hair (Y), according to the formula Y=X*D*101/5, where D is the dilution factor (in the absence of dilution D=1).

3.4.3. Serological Studies

The seropositivity of animals to various pathogens was assessed using ELISA kits. The evaluation was performed using commercial kits of IDVet (Netherlands) (antibodies to Trichinella sp.) and Chema (Russia), (antibodies to mobiliviruses, mycoplasma, chlamydia, toxoplasma, herpes simplex virus, Candida sp., Brucella sp., Trichinella sp.). Protein A conjugated with horseradish peroxidase was used as a conjugate in the Chema kits. Protein A has a high affinity for immunoglobulins, which allowed the detection of antibodies in Caspian seal that bound to the immobilized antigen on the plate surface. Determination of seropositivity to each of the eight pathogens tested was performed for 20 samples of Caspian seal sera collected in 2023.

Seventy-two sera collected from Caspian seals between 2019 and 2023, were tested for the presence of influenza antibodies using the IDEXX Influenza A Ab Test Cat.# 99-53101 ELISA kit according to Bodewes et al. Antibodies (IgG) to carnivore morbilliviruses in animal sera were analyzed using the CDV (Distemper Virus) IgG antibodies ELISA Cat.# DE2478 kit according to the manufacturer's instructions and by virus neutralization.

3.4.4. Protocol of Sample Processing for Mmorphometric Studies

Sample preparation protocol for light microscopy

The material was washed from foreign contaminants in detergent solution. Fangs and claws were photographed using a Keyence VHX-1000 universal digital microscope (Keyence Corporation, Japan) at magnifications of x20 to x30 using the 2d image stiching function. Measurements of claw and fang parameters were performed in the standard software installed in the control computer of the microscope.

Next, whole fangs and claws were sawed on a TechCut 4 low-speed cutting machine (Allied High Tech, USA) using a standard cutting disk with resin-based diamond abrasive (disk brand: RB, D). The canines were cut transversely at the crown-root interface, and the claws were cut longitudinally from base to tip. The finished cuts were manually polished sequentially on three Allied diamond lapping disks (diamond lapping film) with 9, 3 and 1 micron grit respectively. The surface of the sawed fangs and claws was microscopically photographed using a Keyence VHX-1000 universal digital microscope (Keyence Corporation, Japan) at magnifications of x20 to x30 and x200 using the 2d image stiching function. To increase the contrast of individual layers on the spires, their surface was covered with water or water-glycerol solution.

Sample preparation protocol for scanning electron microscopy

Sawed fangs and claws were attached with electrically conductive carbon tape to standard SEM tables with the ground surface facing upward and placed in a Q150R ES Plus (Quorum Technologies, U.K.) to evacuate the gases and water contained in them for three hours.

The obtained preparations of canines and claws were sputtered with gold on a S150A Sputter Coater (Edwards, U.K.) and examined on a MIRA 3 LMH scanning electron microscope (TESCAN ORSAY HOLDING, Czech Republic) equipped with an AZtecOne X-act energy dispersive microanalysis system. Fangs and claws were photographed using the SEM.

For fangs and claws, photographs of some areas of interest for analysis were obtained at high magnification.

Protocol for light microscopy and morphometry

Collected material was photographed using light microscopes: claws and fangs. –via a Keyence VHX-1000 microscope (Keyence Corporation, Japan). The length and width of the objects were measured. After taking general views, claws and canines were cut longitudinally (two canine specimens were also cut transversely) on a TechCut 4 machine (AlliedHighTech, USA), the surface of each cut was polished and photographed on a Keyence VHX-1000 microscope (Keyence Corporation, Japan). The necessary morphometric measurements were performed.

Protocol for electron microscopy and morphometry

The surface of sawed claws and canines was cleaned from foreign particles in detergent solution and air-dried. Cleaned claws and canines were glued to electron microscopy tables with the polished surface upwards using varnish, sputtered with gold on a Q150R ES Plus machine

(QuorumTechnologiesLtd, UK) and photographed using a MIRA 3 LMH scanning electron microscope (TESCAN ORSAY HOLDING, Czech Republic).

3.4.5. Microbiological Studies. Viral Genome Isolation

The samples were thawed before DNA isolation. One of the three sampled swabs was used for metagenomic work. For DNA isolation, a set of reagents NucleoSpin Soil, MACHEREY-NAGEL (Germany) was used according to the manufacturer’s instructions.

The quantity and quality of isolated DNA was determined using a Qubit fluorimeter, Thermo Fisher Scientific (USA). Amplicon libraries were prepared by PCR with primers on V3-V4 regions of eubacterial 16S rDNA: F (341) 5'-CCTACGGGGGNGGCWGCWGCAG-3' / R (805) 5'-GACTACHVGGGTATCTAATCC-3'.

Amplicon libraries were sequenced on the Illumina MiSeq 2 × 300 bp Illumina MiSeq platform using the equipment of the Genomic Technologies, Proteomics, and Cell Biology Center of FSBSI “RRIFO” (VNIRO). Raw reads (reids) were processed in the Qiime environment with the cutadapt algorithm to remove adapters, primers, and primerless reids. The resulting sequences were trimmed until Q25 quality level ≥ 100% and paired using the DADA2 algorithm. Chimeras were removed using the Qiime-Vsearch algorithm. The remaining sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity criterion (open reference algorithm) and then annotated against the Silva132 database using a 97% similarity criterion. The adequacy of OTE annotation was manually checked against the Genbank PubMed database (Blast algorithm), and outdated taxonomy was corrected. Unannotated “de novo” OTUs were also manually annotated against the Genbank PubMed database (Blast algorithm). OTUs with identical annotation were demultiplexed.

The functional capabilities of microbiomes were evaluated using the PICRUSt package using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [Langille et al., 2013]. Values are presented in normalized form.

To obtain full-length 16SrRNA genes of key microorganisms, shotgun sequencing of total DNA was performed on the Illumina HiSeq platform with a read depth of at least 5GB. Processing of the obtained reads was carried out according to the following steps: 1. Preprocessing (Trimmomatic and CutAdart scripts), 2. Quality check: (FastQC complex), 3. Combining reids, removing short reids (PEAR, Qiime complexes), 4. Contig assemblies and quality assessment (MetaSpades), 5. Annotation by databases (Blast, MEGAN algorithms).

Basic statistics (mean and standard deviation), nonparametric ANOVA and calculation of alpha diversity indices were calculated in the Qiime environment.

3.4.6. Immunity Studies

The immune status of animals was assessed using four main methods: hemoagglutination reaction to evaluate the concentration of available nonspecific antibodies (natural antibodies), evaluation of lysozyme concentration and total immunoglobulin G concentration, and bacteria killing assay. Most studies in laboratories focus on the evaluation of parameters of the immune response of animals to certain stimuli, such as the introduction of non-replicating antigens (ram's erythrocytes, hemocyanin, etc.) [Naidenko et al., 2019].

Serial dilution of serum samples with saline in 96-well U-bottom plates was performed (50 µl serum + 50 µl saline, titer 1:1, 1:2; 1:4... 1:1024). 50 µl of ram erythrocyte solution with a concentration of 100x106 units/mL was added to each well and then incubated for 90 minutes at T=37°C. The last well in which no precipitate of ram erythrocytes was formed was taken as the final titer. The assay results were logarithmized (log2).

lysozyme concentration in animal serum was MEsured based on the method of Heinrich et al. [Heinrich et al., 2017]. The range of standards was from 0 to 500 µg/mL. 1% agar was prepared on sterile PBS with pH modified to 6.3, cooled to 50-60°C, then lysozyme sensitive bacterium Micrococcus lysodeikticus (Sigma Aldrich; M3770) was added with a concentration of 40 mg/100 ml, and poured into a 96-well plate at 250 µl/well. After agar solidification for one hour, 20 µl of lysozyme standards or test sera were added to each of the cells. Incubation was 18 hours at room temperature, after which optical density was read using a flatbed spectrophotometer at a wavelength of 350 nm.

Total immunoglobulin G concentration was measured according to the generally accepted methodology [Heinrich et al., 2017]. A linear standard curve was calculated for each plate using standard concentrations. IgG concentrations were then calculated based on the standard curve.

Serum activity in the bacterial killing test was performed according to the method described above (Heinrich et al., 2016). The test results were evaluated according to Heinrich et al., 2016, taking as a result the titer (and subsequently the dilution) at which the serum killed 50% of the bacteria. If this was not observed even in the first cell (at titer 2 and dilution number 1), the test result was taken as 0.

3.4.7. Virological Studies (SPC M&V. RoK)

3.4.7.1. Protocol for isolation of viral genome from biological samples and synthesis of complementary DNA chain

Organ and tissue samples were grinded in cryotubes with transport medium using stainless steel balls in an automatic high-speed closed-type homogenizer TissueLyser II (QIAGEN).

The resulting homogenates were further processed using a QIAshredder (biopolymer shredder in microcentrifuge column format) to improve nucleic acid yield, by plating 700 µl of samples each on the columns of the system. Samples were centrifuged for 2 min at 14000 rpm. The resulting tissue lysates were used for virus nucleic acid isolation procedures.

RNA extraction from organ homogenates was performed using TRIZOL LS reagent (Invitrogen), according to the manufacturer's recommendations from 50-100 mg of tissue or 250 µl of organ suspension [Ausubel et.al., 1990; Chomczynski and Sacchi, 1987]. To 250 µl of lysate, 750 µl of TRIZOL LS reagent was added and incubated for 5 min at room temperature to dissociate the nucleoprotein complex. Then 200 µl of chloroform was applied and the suspensions were centrifuged to separate into aqueous and organic phases. Total RNA was extracted from the aqueous phase by precipitation with isopropyl alcohol.

RNA isolation from swabs was performed using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden), according to the manufacturer's recommendations from 140 µl of swab [QIAamp Viral RNA Vini Handbook, 2005].

3.4.7.2. Protocol for reverse transcription and polymerase chain reaction (RT-PCR)

RT-PCR was performed simultaneously in a one-step reaction using OneTaq RT-PCR Kit (New England Biolabs, Ipswich, MA, USA) [Payungporn et.al., 2004]. To prepare the reaction mixture, a set of primers specific to highly conserved regions of the genes of the desired viruses (Table 3.4-2) was used in a final volume of 25 µl.

The reaction was performed in an Eppendorf Gradient thermocycler under the following conditions: reverse transcription at 48°C for 45 min, initial 2 min denaturation at 95°C, and amplification in 40 cycles including denaturation, primer annealing, and chain elongation (Table 3.4-2, 3.4-3) followed by final elongation at 72°C for 10 min.

Table 3.4-2 Sequences of Specific Primers to Virus Genes

Target gene	Sequence of primer	Length of expected PCR product (bp)
M influenza A virus gene	CU-MF: 5'-TGATCTTCTTGAAAATTTGCAG-3' CU-MR: 5'-TGTTGACAAAATGACCATCG-3'	279
NS influenza B virus gene	NS1: 5'- ATG GCC ATC GGA TCC TCA AC-3' NS2: 5'- TGT CAG CTA TTA TGG AGC TG-3'	240
PB1 influenza D virus gene	5'-TGGATGGAGAGTGCTGCTTC-3', IDV R reverse: 5'-GCCAATGCTTCCTCCCTGTA-3', IDV Probe FAM-CATGTTAAACATTCCCATCAGCATTCCT_BHQ1	110
P gene of morbillivirus	UP-P1:-5' ATGTTTATGATCACAGCGGT-3' UP-P2: -5' ATTGGGTTGCACCACTTGTC-3'	429
L gene of the paramyxovirus family	PAR F1:-5'GAAGGITATTGTCAIAARNTNTGGAC-3' PAR F2:-5'GTTGCTTCAATGGTTCARGGNGAYAA-3'	600
poly gene of the coronavirus family	Cor-FW:- 5'ACWCARHTVAAYYTNAARTAYG-3' Cor-RW:- 5' TCRCAYTTDGGRTARTCCC-3'	251
poly gene of the adenovirus family	polFouter [5'-TNMGNGGNGGNMGNTGYTAYCC-3'; polRouter [5'-GTDGCRAANSHNCCRTABARNGMRTT-3', - for round two polFinner (5′-GTNTWYGAYATHTGYGGHATGTAYGC-3′ polRinner (5′-CCANCCBCDRTTRTGNARNGTRA-3′).	318-325
gp64 gene of herpesviruses	F 5'-CCGCAACCTGGTGCCCATGG-3 R 5'-CGTTTGGGTTGCGCAGCGGG -3	139

Target gene	Sequence of primer	Length of expected PCR product (bp)
gD gene-herpesviruses	seal herpes gD1 (F) 5 - GAAGTTCGGTATGTWAC - 3, seal herpes1 gD2 (R) 5 - AATCCCAATTCATCRTC - 3.	290
Retroviruses	Pan-retrovirus F 5′-TGGAAAGTGYTRCCMCARGG-3′, Pan-retrovirus R 5′GGMGGCCAGSAKGTCATCCAYGT-3′	1300

Table 3.4-3 PCR Thermocycling Parameters with Different Primers

A couple of primers	Chain denaturation		Primer annealing		Chain elongation	
	Temperature	Time	Temperature	Time	Temperature	Time
CU-MF/MR	94°C	30 sec	55°C,	30 sec	72°C,	30 sec
NS1/NS2	95°C	1 min	45°C	1 min	72°C	3 min
P1/P2	95°C	1 min	53°C	1 min	72°C	1 min
PMX1/PMX2	95°C	15 sec	41°C	30 sec	72°C,	30 sec
PAR F1/ PAR F2	94°C	15 sec	50°C	30 sec	72°C,	30 sec
Cor-FW/ Cor-RW	94°C	30 sec	48°C	30 sec	72°C	1 min

3.4.7.3. Protocol for Electrophoretic Analysis of PCR Products

Horizontal gel electrophoresis was performed in 2% agarose solution (Sigma, USA) stained with ethidium bromide in Tris-acetate buffer at 88V (8 volts/cm) on a Biostep apparatus (UK). Visualization and documentation of gel electrophoresis results were performed using a GelMax® 125 Imager system (Upland, CA, USA).

3.4.8. Sequencing and metagenomic data analysis

For massive parallel sequencing, libraries were constructed using the NEBNext Ultra DNA Library Preparation Kit (New England Biolabs, USA) according to the manufacturer's protocol, library size selection was performed using Ampure XP beads (Beckman Coulter, USA), and libraries were checked on a Bioanalyzer 2100 (Agilent Technologies, Germany). Sequencing was performed using MiSeq Reagent v.3 kit on a MiSeq sequencer (Illumina, USA).

Data was processing using a Linux machine located locally using the DIAMOND + MEGAN analytical combination. The initially raw metagenomic data obtained from each pool were preprocessed using Fastp software (version 0.20.0) with the following parameters: "-n 0 -l 30 -5 -r -W 5 -cut_mean_quality 20". This preprocessing step was aimed at excluding low-quality tails and sequencing adapters. In this step, the total number of clean reads obtained from the four pools was determined. To ensure contamination control, sequencing reads were aligned to the Pusa caspica genome using the BWA-mem v. 0.7 tools and subsequently removed from the dataset.

Subsequently, quality-controlled reads were reassembled using MEGAHIT v1.2.9 with "-k-min 21 -k-max 141 -k-step 20" [Green, K.Y. et al., 2000]. For taxonomic annotation of the collected contigs, a comparison was made with the NCBI number database (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz) (as of May 10, 2023) using DIAMOND Blastx v. 0.9.24. The threshold E value used was $<10^{-5}$ [Smith A.W. et al., 1973]. Subsequently, contigs were taxonomically classified and results were visualized using default settings in MEGAN (MEtaGenome Analyser, version 6.24.23, Tübingen, Germany) [Gulland F.M. et al., 1997; Smith A.W. et al., 1973]. MEGAN results, which were generated as summarized reads, were used to present a comprehensive overview of the taxonomic classification of sequencing reads. Exclusion criteria were applied to contigs originating from cellular organisms, including bacteria, archaea, and eukaryotes.

To exclude false positive virus hits, further analysis of virus-like reads was performed using DIAMOND Blastx v. 0.9.24 (E value < 10-10) and compared to the NCBI virus reference sequence database (RefSeq) (https:// ftp.ncbi.nih.gov/refseq/release/viral/) to identify sequences with higher similarity to viral sequences compared to non-viral sequences. The obtained results were further analyzed using MEGAN software (v. 6.24.23) with the least common ancestor (LCA) parameter. A minimum threshold score of 100 and an e-value threshold of 1×10^{-10} were applied during the analysis.

PCR products were sequenced by Sanger sequencing and compared with the original matrix. Phylogenetic analysis was performed using the neighbor-joining method with 1000 bootstrap repeats using the p-distance model in the MEGA X software [Kumar S. et al., 2018].

4. FIELDWORK RESULTS IN 2023

4.1. Meteorological Observations

Meteorological parameters are provided in Appendix C, Table C.1. Measurements were carried out by the multi-attribute Skywatch GEOS meteorological station.

Air temperature. The weather was stable at the time of the survey work. Morning air temperatures ranged from +2 to +70C, daytime temperatures ranged from +7 to +160C, and evening temperatures ranged from +8 to +13°C. Mean daily temperatures ranged from 5.8 to +10.50C. The graph of average daily air temperature is shown in Figure 4.1.1.

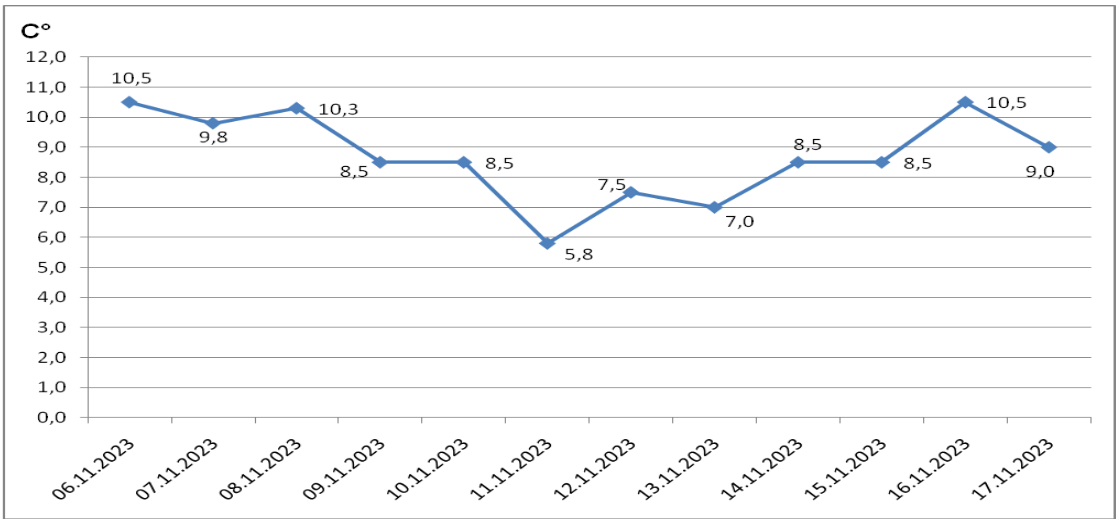


Figure 4.1.1 Graph of the Average Daily Air Temperature, November 2023

Atmospheric pressure. During the 2023 survey period, the atmospheric pressure was in the range of 762 – 776 mm/Hg. It depends on the season of work, air temperature and influence of air masses. The graph of the mean daily atmospheric pressure is presented in Figure 4.1.2.

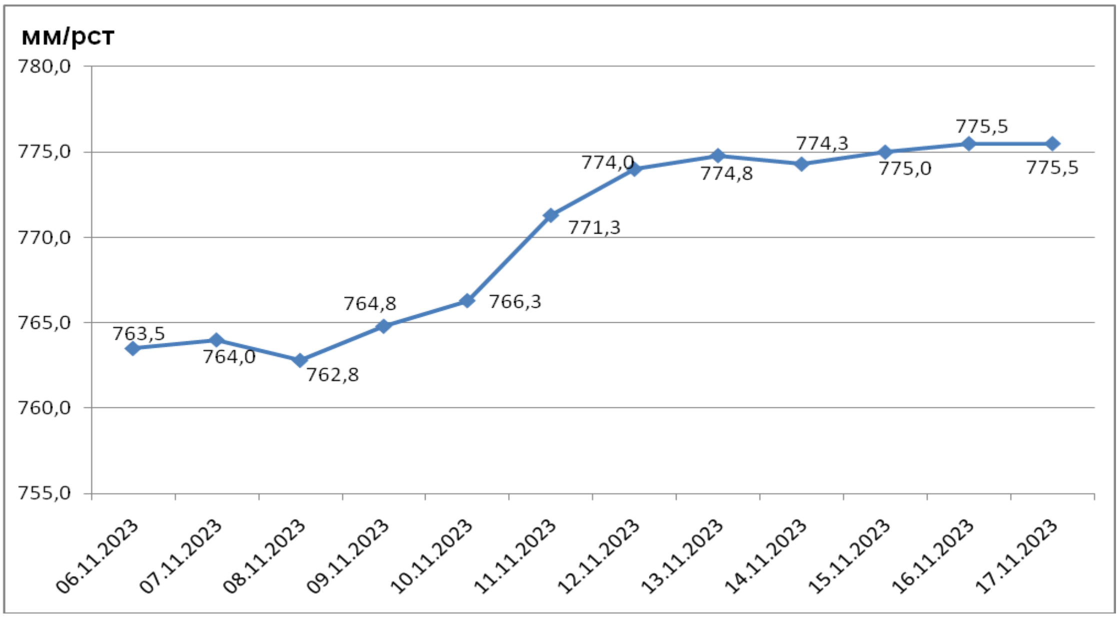


Figure 4.1.2 Graph of the Average Daily Atmospheric Pressure

Wind direction and speed. In autumn 2023, during the works, the weather was not stable, with mainly western and south-eastern wind directions. Wind speeds ranged from 1 to 14 m/s and gusts up to 18 m/s, as shown in Figure 4.1.3.

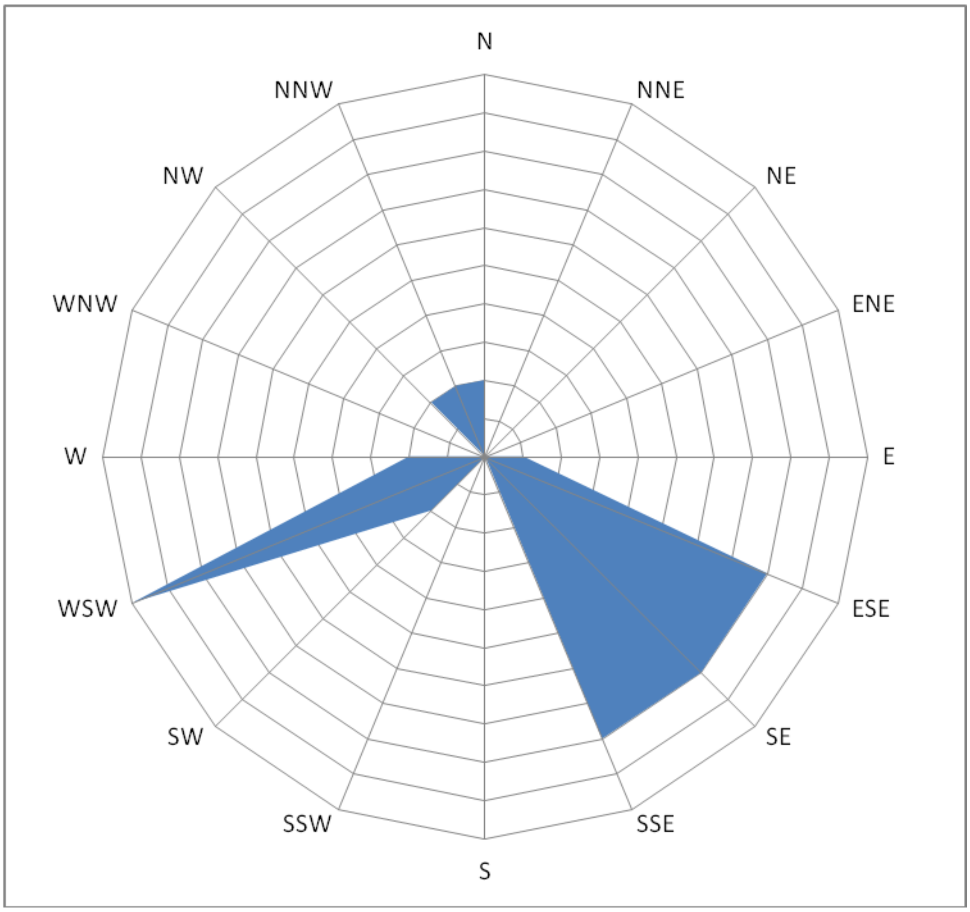


Figure 4.1.3 Windrose during Survey Period

4.2. Record of Seal Abundance

During the voyage of the R/V "Nautilus-One" (07.11.2023-16.11.2023) in the Kazakhstan water area of the Caspian Sea, 200 sightings of Caspian seals (217 individuals) were recorded (see Figure 4.2.1). In preparation for seal capture, six sand islands were inspected, with four of them (KT1 - KT4) being submerged due to surge and sea level rise (refer to Table 4.2-1). Caspian seal haul-outs were recorded at KT5 and KT6, and 9836 Caspian seals were counted during two days of trapping. Additionally, four dead seals were recorded on the way to the KT2 sand island.

Table 4.2-3 presents the calculation of the seal density encountered in the Kazakhstan part of the Northern Caspian Sea.

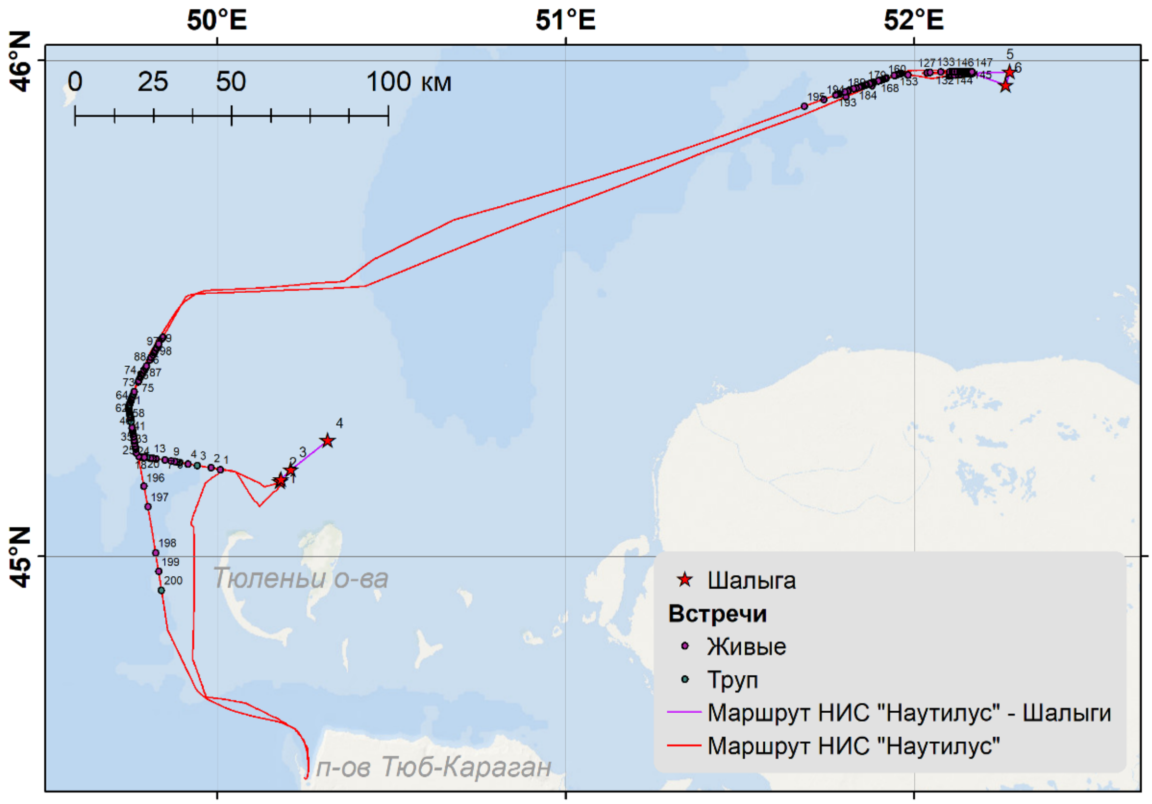


Figure 4.2.1 Layout of seal sighting points along the ship route.

Table 4.2-1 Coordinates of surveyed sand islands in November 2023.

No.	Sand island	North longitude	East longitude
1	Submerged elongated sand island	45.15253°N	50.18067°E
2	Recessed elongated E-shaped sand island	45.156176° N	50.183407° E
3	Trapezoidal sand island	45.17670°N	50.21097°E
4	Submerged elongated sand island	45.23598°N	50.31708°E
5	C-shaped small sand island (NCMC)	45° 58' 39.32"N	52° 16' 28.94"E
6	Long sand island submerged in some areas (NCMC)	45°57'3,35"	52°15'50,40"

Table 4.2-1 Numbers of Counted Seals, Nautilus-One SRV, November, 2022

Date	Observer	Time	Number of specimens	Distance from vessel, m	Comments
11.11.2023	FC AM	12:00-13:00	18	20-400	The number of seals on the boat route was counted, 14 live and 4 dead. The route length was 34 km. The survey width was 400 m on each side. No seal haul-outs were found on 4 sand islands (KT1-KT4), as well as on Podgorny, Morsky and Kulaly islands.
11.11.20223	GP NP SE PI	14:00- 18:00	147	20-400	Observations from the R/V Nautilus One. Northeast Caspian Sea, distance 217 km. Observations from port and starboard sides. Strong swell, wave 60-110 cm
13.11.2023	FK AM	15:00-17:00	46	20-400	A count of the number of seals on the route of the boat. of the boat's movement, 46 seals in total, of which 37 from the port side and 9 from the starboard side. The route length was 24.6 km. The survey width was 400 m on each side.

Date	Observer	Time	Number of specimens	Distance from vessel, m	Comments
	AM AM	17:32	278		A survey of KT5 sand island was conducted using a quadrocopter. The flight height was 150 m. There were 278 seals on the KT5 sand island.
	AM AM	17:54	9558		A survey of KT6 sand island was conducted using a quadrocopter. The flight height was 100m. There were 9558 seals on the KT6 sand island. 13 seals were caught on the sand island and 13 radio beacons were installed.
14.11.2023	FK AM	11:00-12:00	60	20-400	The number of seals was counted on the way of the boat, a total of 60 seals, of which from the port side 32 seals, from the starboard side 32 seals. 32 seals on the port side and 28 seals on the starboard side. The route length was 8.0 km. The survey width was 400 m on each side. 7 Caspian seals were caught on the KT6 sand island.
16.11.2023	GP NP PS PI	9:00- 12:00	70	20-400	Observations from the R/V Nautilus One. Northeast Caspian Sea, distance 118 km. Observations from the port and starboard sides. Strong swell, 80-100 centimeters wave
Total:			**10177**		**4 of them dead**

Note: *GP - Gleb Pilipenko, NP - Nikita Platonov, PS - Polina Shibanova, PI - Polina Ilyina, FK - Fedor Klimov, AM - Alexey Mulyaev.*

Table 4.2-2 Density of Seals Distribution in the Kazakhstan part of the North Caspian Sea, 2023

Date	Place	Route length, km*	Width of the recording	Area of recording strip, km2	Quantity of accounted seals, specimens	Density, specimens/km2
11.11.2023	Nautilus-One	217	1	217	147	0,68
16.11.2023	Nautilus-One	118	1	118	70	0,59
13.11.2022	Counting on sand island KT5	0,300	0,1	0,03	278	9267
13.11.2022	Counting on sand island KT5	1	0,05	1	9558	9558
11.11.2023	Motor-boat	34	0,8	27,2	14	0,66
13.11.2023	Motor-boat	24,6	0,8	19,7	46	4,5
14.11.2022	Motor-boat	8,0	0,8	6,4	60	9,4
Average value recorded from boat						**4,85 (from boats)**

Note: ** total length of the route segments where observations were made*

Studies have shown that the number of seals in areas with depths of 5-12 m ranges from 0.59 to 0.68 individuals per square kilometer. Above depths of 0.5-4.0 m, the range is 0.66-9.4 individuals per square kilometer, with the highest noted in recreational areas and sand islands at 9267-9558 individuals per square kilometer.

4.3. Caspian seal survey in 2023.

Estimation of the number of seals on sand islands in the Kazakhstan sector of the Caspian Sea was carried out using a DJI Mavic 2 Pro quadrocopter. During the preparation for seal capture 6 sand islands were inspected, 4 of them (KT1-KT4) were submerged due to surge and water level rise. Seal haul-out was observed only at KT5 and KT6. When live seals were captured at KT6, the number of seals at the haulout was 9267, of which 20 seals were captured for sampling during 2 days.

The demographic structure of the Caspian seal population (the ratio of males and females of different ages) was studied in parallel with sampling of their genetic material and other samples at the seal deposition sites.

On November 5-6, 2023 from the Department of Fishery Inspection in Mangistau Oblast it was reported about the release of 40 dead seals on the shore, in the area between the villages of Bautino and Fort Shevchenko. Employees of the laboratory of virus ecology of NPC MIV (RK) together with scientists of KAPE LLP in the presence of representatives of environmental structures of Mangistau region participated in the study of the causes of death of animals. For this purpose, the dead animals were examined for virological and bacteriological analyses. The degree of decomposition of dead animals was high, dead animals were not suitable for sampling, as autolysis processes mask the picture of pathological changes.

4.3.1. Age and Gender Composition

Data on live seals are presented in Appendix C, Table C.2. The majority of seals were represented by females (92%), with only two males (8%). Since no in vivo methods of age identification were developed for the Caspian seal, the sample list of live animals was subdivided into several age groups according to the number of complete segments on the claws (Table 4.3-1).

Table 4.3-1 Number of Captured Live Seals in Different Age Groups

Age group	Number of specimens	Ratio, %
youger than three years old	5	25
over 3 years old (3+)	2	10
over 4 years old (4+)	3	15
over 5 years old (5+)	5	25
over 6 years old (6+)	3	15
over 7 years old (7+)	1	5
over 8 years old (8+)	1	5

4.3.2. Damage to Skin, Hair Cover, and Other Visual Abnormalities

Visual abnormalities deviating from the normal physiological condition were detected in 11 live seals (Appendix C, Table C.3), representing 55% of the total number of live individuals examined. The majority of cases can be attributed to the following types of abnormalities: eye diseases, absence of claws, skin and hair lesions (Figure 4.3.1).

Missing Claw (PC-23-18)

Wound on the Right Foreleg (PC-23-18)

Anal Irritation (PC23-04)

Presence of Bald Patches (PC23-09)

Figure 4.3.1 Skin-Hair and Other Abnormalities in the Caught Seal

Body wounds were found on seals PC23-04, PC23-06, PC23-10, and PC23-11. Two seals, PC23-08 and PC23-17, exhibited eye diseases: PC23-08 had greenish-yellow discharge from the eyes, while PC23-17 showed irritation of the mucosa.

Three seals, PC23-08, PC23-09, and PC23-18, were observed to have one or more claws missing. Additionally, PC23-11 had a broken claw, and PC23-12 had a damaged claw. Some animals showed signs of recent injury, as they were bleeding. A few individuals exhibited abnormalities not previously identified. Seal PC23-04 had irritation near the anus, and PC23-09 had bald patches on the skin.

4.3.3. Size and Mass

Live individuals from the 2023 sample were analyzed. As several morphometric traits, particularly zoological length and thorax girth, exhibit seasonal variability, including seals that died at different times of the year could introduce errors in interpreting the research results [Pastukhov, 1993; Ian et al., 2010]. The results of measurements grouped by age are presented in Table 4.3-2.

Table 4.3-2 Size of Seals in Different Age Groups, 2023

Parameter	Age, years							
	1+	2+	3+	4+	5+	6+	7+	8+
Zoological length, cm	94 / 1	88,6±6,2 / 4	92,3±9,2 / 2	121,0±5,6 / 3	121,0±5,5 / 5	122,0±12,1 / 3	117 / 1	120 / 1
Tail length, cm	9 / 1	8,6±1,6 / 4	7,3±0,6 / 2	9,3±0,6 / 3	8,4±1,1 / 5	8,3±2,3 / 3	10 / 1	7 / 1
Chest circumference, cm	87 / 1	80,7±2,9 / 4	94,0±6,2 / 2	121,3±7,8 / 3	121,4±8,0 / 5	123,7±5,1 / 3	117 / 1	125 / 1
Weight, kg	25,5 / 1	21,7±1,9 / 4	29,7±4,0 / 2	74,2±3,9 / 3	73,3±7,2 / 5	73,9±6,2 / 3	56,7 / 1	74,7 / 1

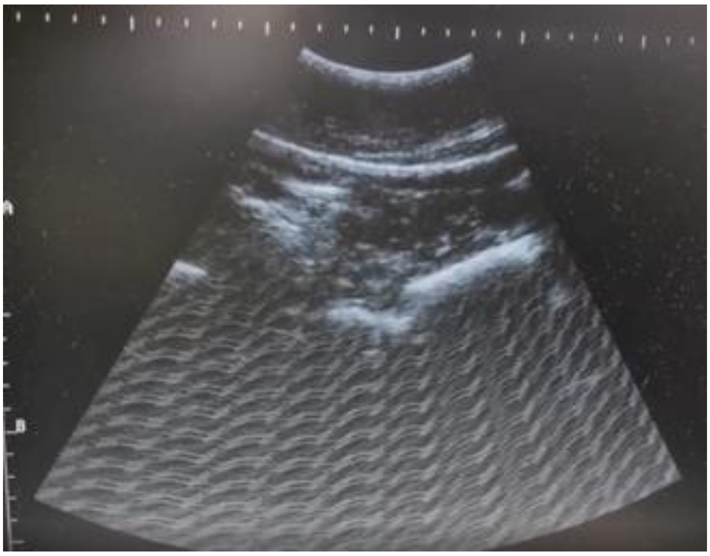
Note: *in the numerator: the mean value ($\bar{x}$) ± standard deviation (σ), in the denominator: the number of specimens (n) in the age group.*

4.3.4. Ultrasound diagnostics (ultrasound, USD)

During the fieldwork, ultrasound scans were conducted on all adult females (PC23-01 to PC23-13) and one young female (PC23-14) to detect the presence of embryos in their abdominal cavities. Based on the ultrasound results (Figure 4.3.2; Appendix C), several groups of females were identified: "pregnant" - females with clearly visible embryo silhouettes; "probably not pregnant" - adult females where the presence of embryos was questionable (silhouette not clearly identified); "very likely not pregnant" - individuals where embryos were not registered; and young animals (ultrasonography was performed only on one individual, PC23-14).

Pregnant adult females (PC23-01)

Adult females that are probably not pregnant (PC23-02)



Young females - high probability of not being pregnant (PC23-14)

Figure 4.3.2 Determination of Pregnancy by Ultrasound Diagnosis

4.4. The Caspian seal movements based on satellite beacon data

The Caspian seal movements based on satellite beacon data are detailed in Appendix C, Table C.4, which presents the beacon installation data.

Out of the 13 tags installed in November, all 13 tags were guaranteed to remain operational as of December 16, 2023 (see Table 4.4-1). However, as of early March 2024, only one tag, No.253218, was still operational (see Table 4.4-2).

Table 4.4-1 Information about Installed Transmitters as of 16.12.2023

Tag No.	Date of the recent signal	Number of locations identified after filtration	Length of track (km)
253210	16/12/23	22	276
253211	15/12/23	58	764
253212	14/12/23	53	795
253213	08/12/23	14	1032
253214	07/12/23	23	548
253215	14/12/23	21	916
253216	15/12/23	27	871
253217	14/12/23	62	1068
253218	11/12/23	43	1152
253219	10/12/23	80	1244
253220	15/12/23	92	1658
253221	14/12/23	55	976
253222	15/12/23	30	1304

Table 4.4-2 Information about Installed Transmitters as of 10.03.2024

Tag No.	Date of the first valid signal	Date of the most recent signal	Number of working days	Number of locations identified after filtration	Length of track (km)
253210	16.11.2023	11.01.2024	56	130	1519
253211	16.11.2023	08.12.2023	22	87	938
253212	16.11.2023	06.02.2024	82	385	3467
253213	16.11.2023	02.02.2024	78	107	3151
253214	16.11.2023	11.01.2024	56	207	2953
253215	18.11.2023	21.01.2024	64	82	1868
253216	16.11.2023	27.01.2024	72	206	1702
253217	15.11.2023	28.01.2024	74	167	2780
253218	19.11.2023	10.03.2024	112	355	2735

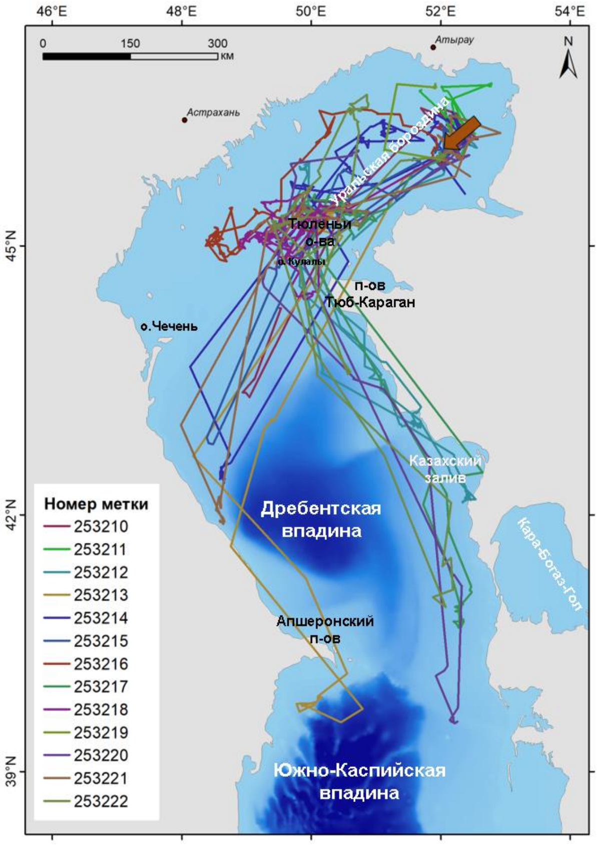
Tag No.	Date of the first valid signal	Date of the most recent signal	Number of working days	Number of locations identified after filtration	Length of track (km)
253219	18.11.2023	26.11.2023	8	57	351
253220	17.11.2023	14.01.2024	58	151	3045
253221	16.11.2023	14.02.2024	90	187	2573
253222	16.11.2023	29.01.2024	74	163	3253

The majority of tags on seals stopped working in January-February. The duration of tagging varied among individuals, ranging from 22 days (tag No.253212) to 112 days (tag No.253218). All tags worked for at least 3 months, except for tag No.253211, which ceased functioning at the beginning of December 2023. Tag No.253219 only transmitted signals from one point in the Ural River delta from December to February, so data from this transmitter for this period were excluded from the analysis.

The frequency of transmitted locations for all tags was regular. An average of 176±98 locations was received from each tag. The highest number of locations was received from tag No.253212, amounting to 385 locations. The lowest number of locations (excluding tag No.253219) was obtained from tag No.253215, totaling 82 locations. Despite the regularity of location receipt, in most cases, the number of days in a month on which locations were received did not exceed 15 (see Table 4.4-3). Figure 4.4.1 shows the tracks of all tagged animals through March 10, 2024.

Table 4.4-3 Number of days per month on which tags transmitted signals

Tag No.	November	December	January	February	March
253210	10	8	4	0	0
253211	10	4	0	0	0
253212	10	16	15	5	0
253213	6	10	10	1	0
253214	9	16	8	0	0
253215	4	12	4	0	0
253216	9	14	11	0	0
253217	7	16	12	0	0
253218	5	14	10	15	6
253219	7	26	24	7	0
253220	10	9	1	0	0
253221	6	9	6	7	0
253222	8	12	1	0	0



Tagging locations are indicated with an arrow

Figure 4.4.1 Tracks of all seals tagged in November 2023 (before 10.03.2024)

Description of movements

November

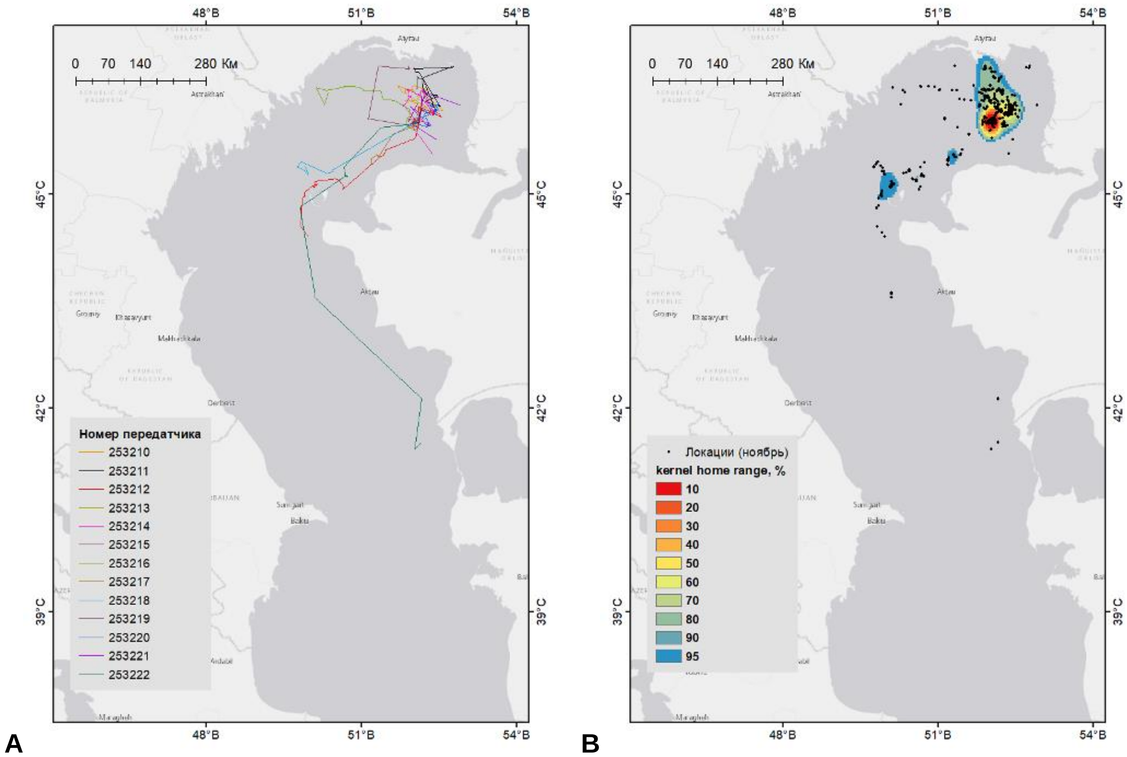
In November 2023, seals mainly migrated within the northern Caspian Sea. The animals were concentrated in the tagging area (Figure 4.4.2). Additionally, seals were sighted in the vicinity of Kulaly Island and the Seal Archipelago Islands. Kulaly Island and the islands of the Seal Archipelago. An animal tagged with No.253222 was also observed in the southern part of the middle Caspian Sea, near Kara-Bogaz-Gol Bay.

December

In December, seals migrated in both the northern and middle Caspian Sea. Animals were still observed in the tagging area, but the primary habitat was established near Kulaly Island and Tyulen Island. Kulaly Island and the islands of the Seal Archipelago are depicted in Figure 4.4.3. Seals tagged with No.253214, No.253213, No.253222, No.253220, No.253212, No.253217 actively utilized the waters of the middle Caspian Sea and ventured into the eastern and western regions of the southern Caspian Sea. Additionally, sightings were reported near the western and eastern shores of the Caspian Sea, including in the vicinity of Kazakh Bay.

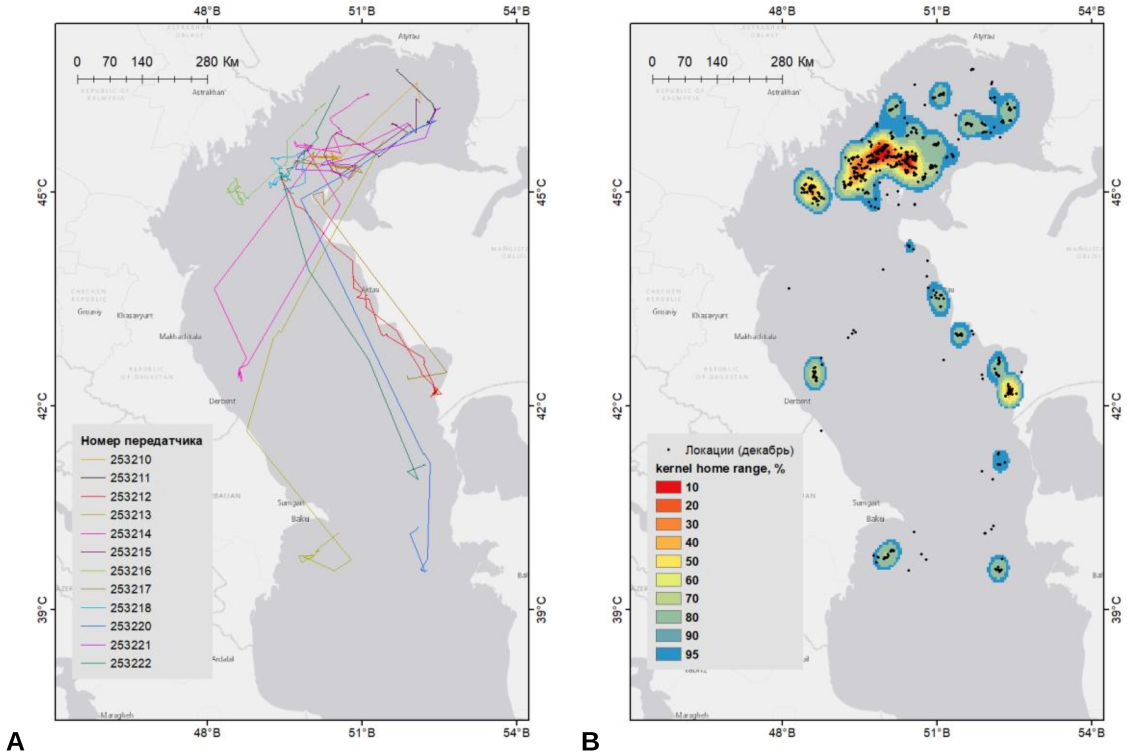
January

In January, similar to December, seals migrated within the northern and middle Caspian Sea. Key habitats were located in the vicinity of Kulaly Island and the Seal Islands. Kulaly Island and the islands of the Seal Archipelago, as well as in the area north of Kazakh Bay (Figure 4.4.4). Seals tagged with No.253221, No.253213, No.253217 were also observed in the southern part of the middle Caspian Sea, moving along the western and eastern shores.



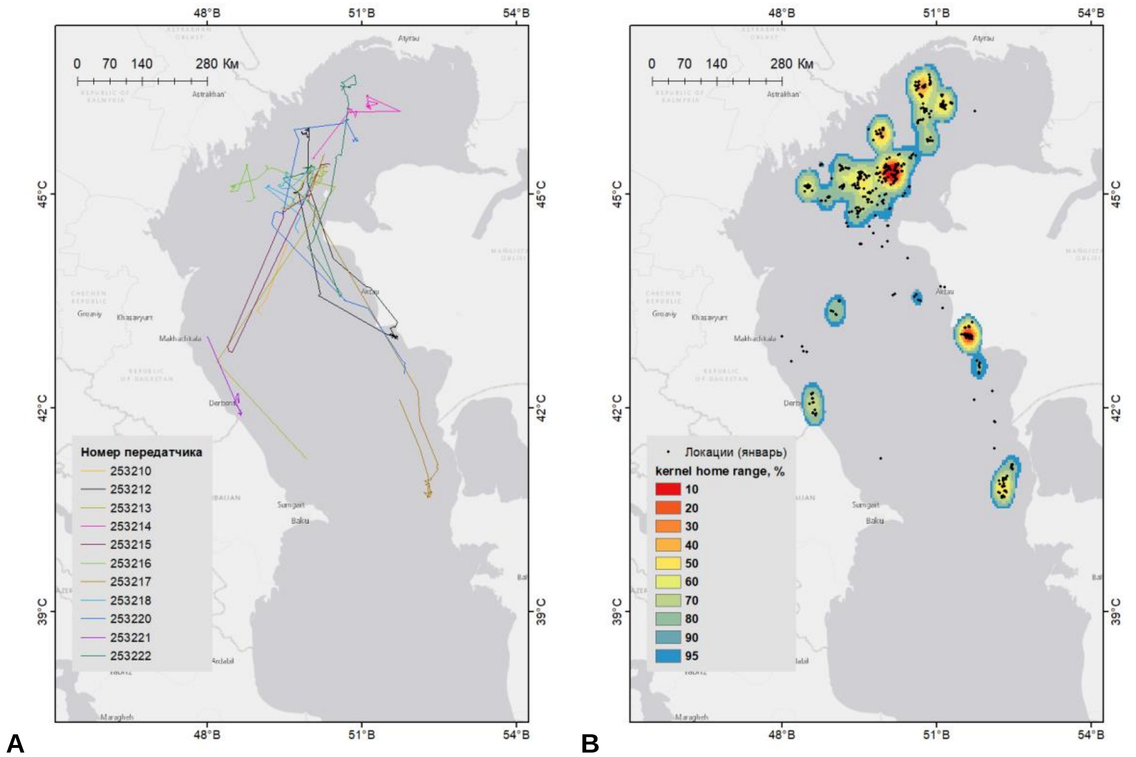
Number of working tags n=13.

Figure 4.4.2 Movement tracks (A) and key seal locations (B) in November 2023



Number of working tags n=13.

Figure 4.4.3 Movement tracks (A) and key seal habitat (B) in December 2023



Number of working tags n=12.

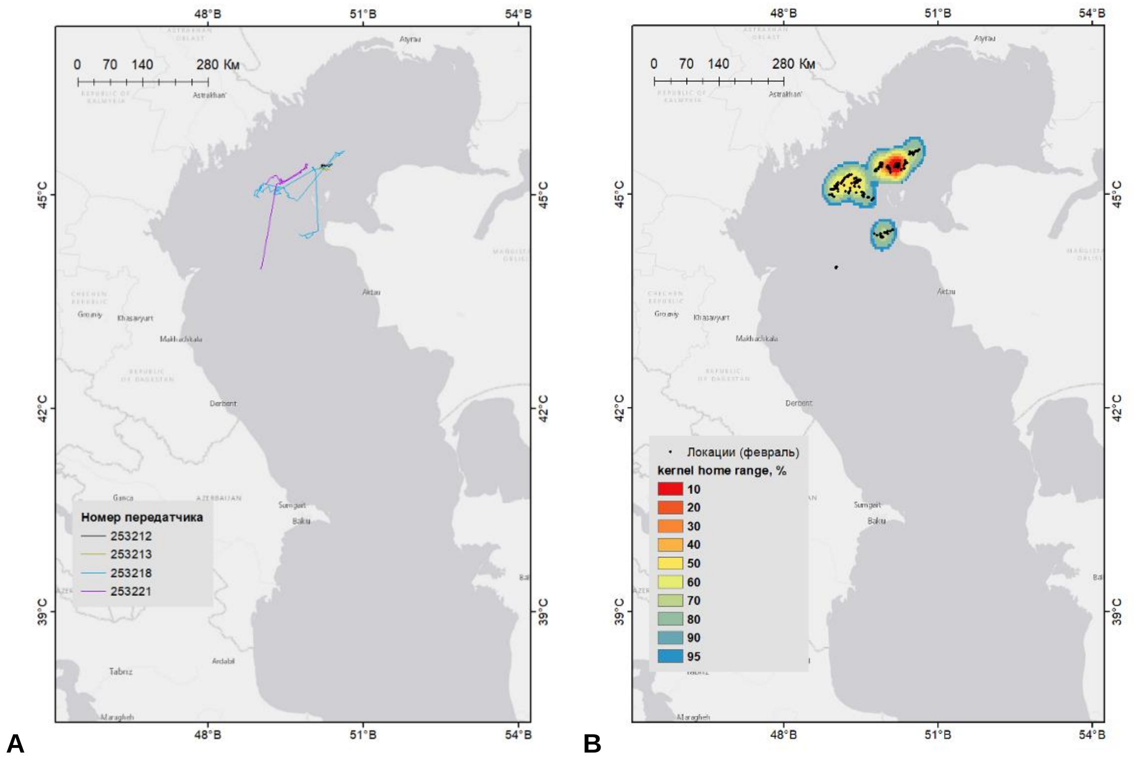
Figure 4.4.1 Movement Tracks (A) and Key Seal Habitats (B) in January 2023

February

In February 2024, signals were received from only four tags: No.253212, No.253213, No.253218, No.253221. Signals from tag No.253213 were received only on February 2, and signals from tag No.253212 were received on February 1, 2, 4, 5, and 6. The seals were located in the northern part of the Caspian Sea, forming key habitats north of Kulaly Island and the Seal Islands. Kulaly Island and the islands of the Seal Archipelago are shown in Figure 4.4.5. Additionally, a seal with tag No.253218 was recorded in the water area near Fort-Shevchenko (Tyub-Karagan (Tupkaragan) Peninsula).

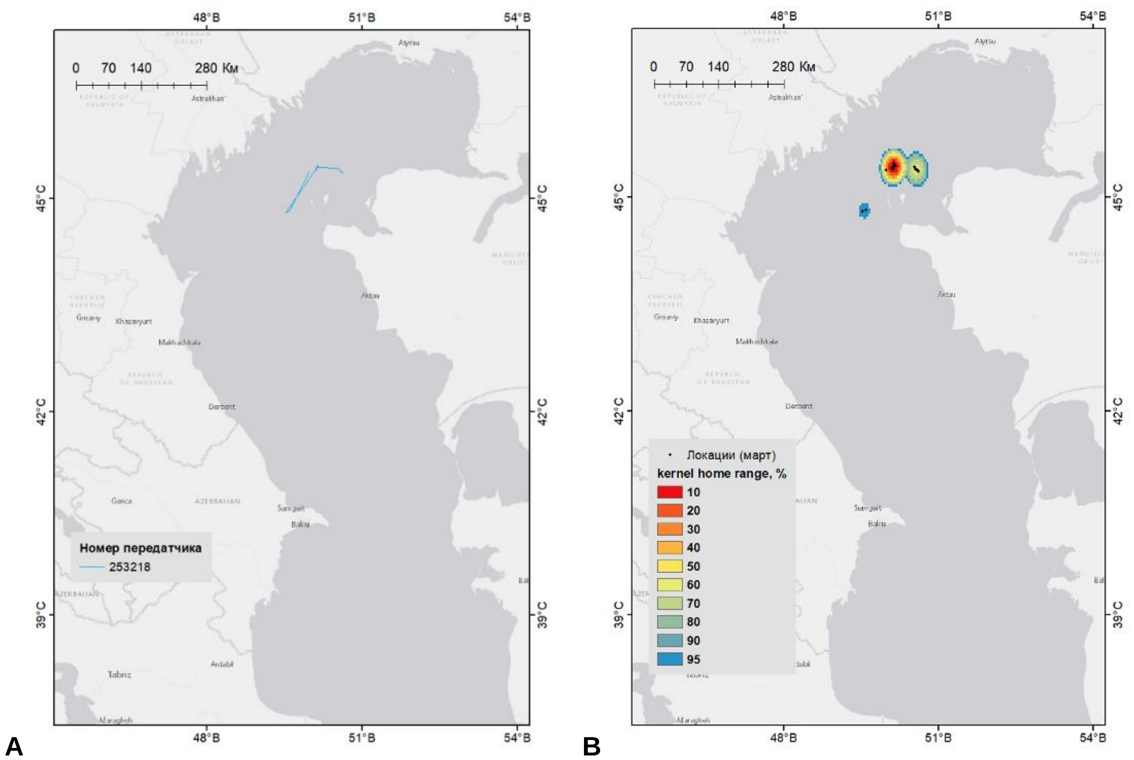
March

In March, the number of working tags decreased, and signals were received only from tag No.253218. An animal, whose transmitter was still working, was in the area of Kulaly Island and the Tyuleny Islands. Kulaly Island and the islands of the Seal Archipelago are shown in Figure 4.4.6.



Number of working tags n=4.

Figure 4.4.2 Movement Tracks (A) and Key Seal Habitats (B) in February, 2024



Number of working tags n=1.

Figure 4.4.6 Movement tracks (A) and key seal habitats (B) in March 2024.

Activity of tagged animals:

The activity of tagged animals from November through March is depicted in Figure 4.4.7. There was a decrease in animal movement activity from December through February. Since only one tag was signaling in March, the distance traveled figures reflect the individual characteristics of the tagged animal whose transmitter was still active in that month.

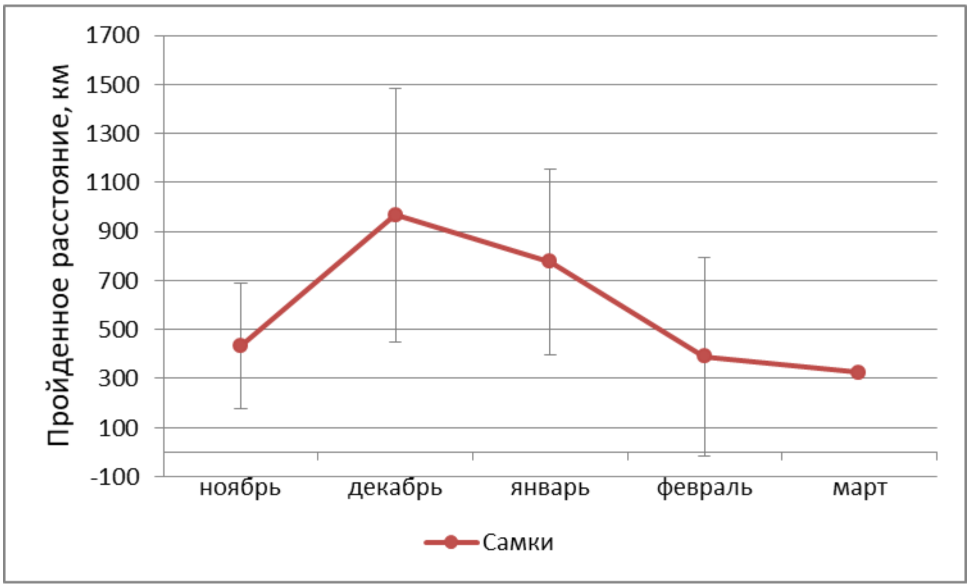


Figure 4.4.7 Activity of tagged animals from November through March

Figure 4.4.8 displays the values of the animal movement speed parameter by month. In November, the rate was slightly lower than in December. From December to March, a gradual decrease in the values of animal velocity is noticeable. It should be noted that this parameter does not reflect the true speed of animal movements, but only the ratio of the distance between two locations according to the tracking data to the number of hours between the two locations.

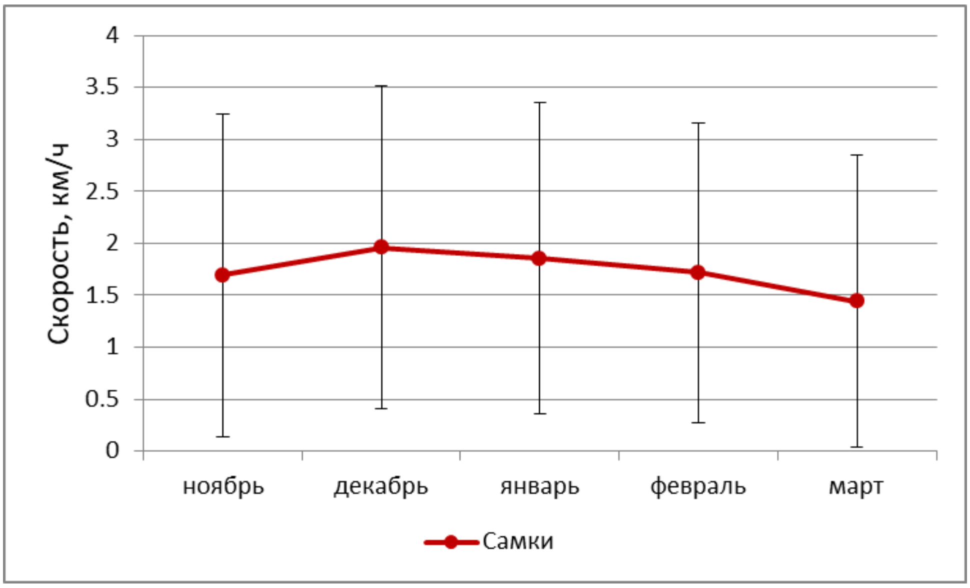


Figure 4.4.8 Speed of movements of tagged animals between November and March (km/h).

The distance to the mainland shoreline of the Caspian Sea, excluding sand islands, is considered the shortest distance to the shore. Changes in the shore distance parameter by month are depicted in Figure 4.4.9. There is a decrease in the median values of the distance to the shore in December and February. However, it should be noted that there is a strong overlap of data scatter, and therefore, we cannot reliably differentiate this parameter by month.

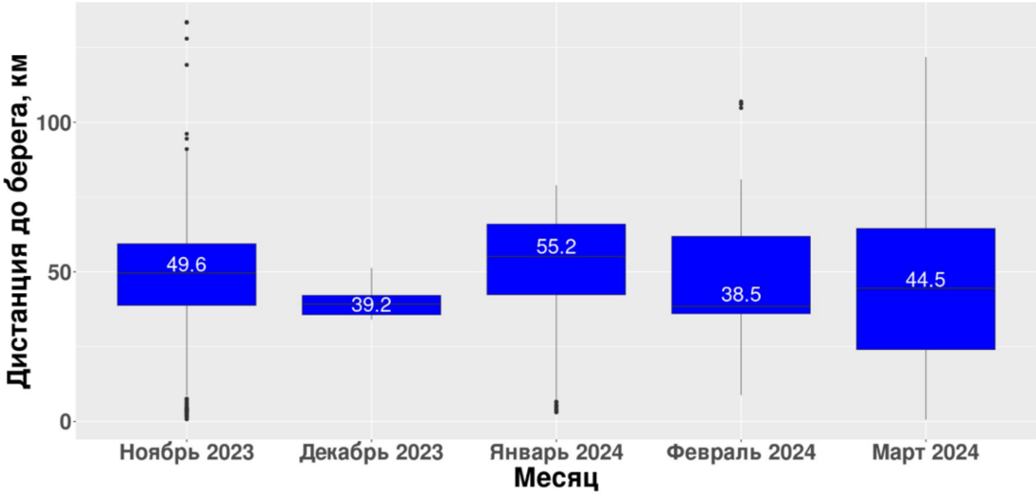


Figure 4.4.9 Distance to shore

The transmitters mounted on the animals did not have a dive depth sensor; therefore, it is not possible to analyze the depth of the animals' dives. However, it is possible to estimate the seals' confinement to water areas with certain depths based on the depth values in the locations. The values of this parameter are illustrated in Figure 4.4.10. Throughout the observation period, the majority of locations were obtained from the northern and middle Caspian Sea, with median depth values for each month ranging from 4 to 6 meters. It is noted that the largest number of locations confined to the deep water areas of the Caspian Sea was obtained in November, which shows a noticeable scatter of data for this month.

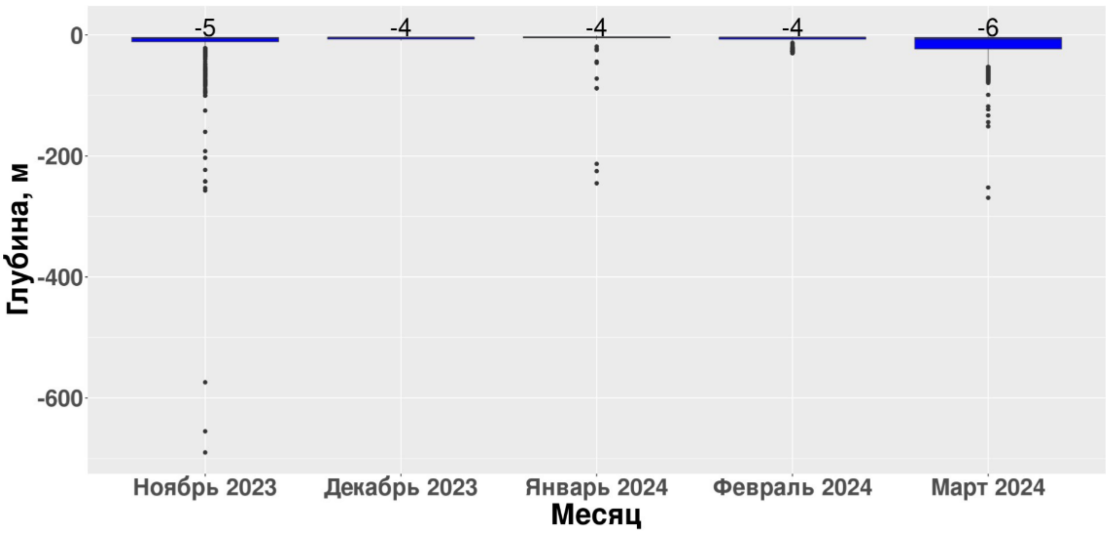


Figure 4.4.10 Depths of water areas where tagged animals were recorded

4.5. Biomaterial Analysis Results

During field work biomaterial samples were taken from live and dead seals (Tables 4.5-1; Appendix B, Tables B.1-B.3).

Table 4.5-1 Biomaterial Samples Taken from Live Seals, November, 2023

Type of sample	The quantity of samples
RPC VM (RK)	
Conjunctival swabs for virological studies	20
Nasal, oral swabs for virological and microbiological studies	40
Urogenital smears for microbiological studies	20
Rectal swabs for virological studies	20
Serum for serological studies	20
IEE RAS (RF)	
Nasal swabs or wipes for virological and microbiological studies	54
Vaginal swabs for microbiological studies	48
Rectal swabs for virological studies	54
Hair samples for hormonal, toxicological studies	60
Vibrissae for hormonal, toxicological studies	40
Blood plasma for serological studies	60
Blood for toxicological studies	40
BGE for toxicological and genetic studies	40
Blood smears for serological studies	40

Specialists from "MiV" (RK) collected 120 biological samples and blood sera from 20 seals for virological, microbiome, virome, molecular-parasitological, and serological studies, without harming the seals' life and health. Additionally, 24 samples of sectional material were collected from deceased seals.

Specialists from "IPEE RAS" (Russian Federation) collected 436 biological samples from live seals for virological, serological, hormonal, genetic, toxicological, morphological, and microbiological studies, without causing harm to their life and health.

4.5.1. Virological and Bacteriological Analysis (RPC VM, RK)

Virological and Bacterial Analysis (NPC MIV RK)

Table 4.5-1 presents characteristics of biological materials collected by specialists from "NPC MIV" (RK) for virological, microbiome, virome, molecular-parasitological, and serological studies. A total of 120 biological samples, including conjunctival, nasal, oral, urogenital, rectal washes, and blood serum, were collected from 20 Caspian seals without causing harm to the animals' life and health. During external examination, some young animals showed symptoms of respiratory infection, such as conjunctival inflammation and catarrhal nasal discharge (Figure 4.5.1).

Figure 4.5.1 Conjunctival inflammation and acute suppurative catarrhal rhinitis in Caspian seals (November 2023).

4.5.1.1. Virological Screening for the Presence of Viral Pathogens Causing Caspian Seal Diseases

The virological screening of 100 samples, including conjunctival, nasal, oral, urogenital, and rectal washes, was conducted on 20 seals to detect pathogens of major viral infections affecting marine mammals and terrestrial carnivores using the PCR method.

The analysis revealed the absence of nucleic acids from pathogens such as influenza (both influenza A and B viruses), coronavirus, paramyxovirus (including carnivore morbillivirus), hepivirus (hepatitis E), poxvirus, lyssavirus, and retrovirus infections in the samples from Caspian seals. These virus families are known to cause widespread infectious diseases in warm-blooded animals. Throughout the study, PCR products corresponding to the expected sizes were only amplified in virus preparations used as positive controls.

In all series of studies, PCR products corresponding to the expected sizes of adenovirus and herpesvirus gene fragments were detected in nasal wash samples collected from animals showing signs of respiratory infection in 2023 (Table 4.5-2). The number of PCR-positive animals for adenoviruses and herpesviruses is depicted in Figures 4.5.2 to 4.5.3.

Adenoviruses were detected in samples from three adult and seven young animals. A BLAST search revealed that the nucleotide sequences of 240 base pairs obtained from PCR sequencing of adenovirus-positive samples matched those of the Caspian seal adenovirus detected in 2016 (98% identity). Further phylogenetic characterization of Caspian seal adenoviruses established their kinship with mink adenovirus (Figure 4.5.4). Further full genomic characterization of Caspian seal adenoviruses is necessary to determine their phylogenetic relationship with other adenoviruses.

Phylogenetically, the closest adenoviruses were those of mink (Lutrine type 1), seals (Phocine 2), and isolates of bat and sea lion mastadenoviruses (Otarine 2), although relationships were implicit. Overall, this suggests the presence of a novel adenovirus in Caspian seals that appears distinct from currently known members of the family Adenoviridae.

Table 4.5-2 Results of PCR screening of Caspian seal samples for the presence of virus genome fragments

Item No.	Type of PCR analysis								
	RT-PCR Influenza A (PB-1 gene)	RT-PCR CDV (P gene)	RT-qPCR CDV	RT-PCR Pan-Paramyxo	Pan-CoV	Pan-Herpes	Pan-Adeno	RT-PCR influenza A (M-gene)	RT-PCR influenza but B (NS-gene)
1	–	–	–	–	–	+	–	–	–
2	–	+	?	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–
4	–	+	?	–	–	–	–	–	–
5	–	+	?	–	–	–	+	–	–
6	–	–	–	–	–	+	+	–	–
7	–	–	–	–	–	+	+	–	–
8	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	+	–	–	–
11	–	–	–	–	–	–	–	–	–
12	–	–	–	–	–	+	–	–	–
13	–	–	–	–	–	–	–	–	–
14	–	–	–	–	–	?	+	–	–
15	–	+	?	–	–	+	+	–	–
16	–	–	–	–	–	+	–	–	–
17	–	–	–	–	–	+	–	–	–
18	–	–	–	–	–	+	–	–	–
19	–	–	–	–	–	+	+	–	–
20	–	–	–	–	–	+	+	–	–
21	–	–	–	–	–	+	+	–	–
22	–	–	–	–	–	+	+	–	–
23	–	–	–	–	–	+	–	–	–
24	–	–	–	-	–	+	–	–	–
Positive Control	Variety (7994)	Nobivac & Biocan DHPPi+LR vaccine	Nobivac & Biocan DHPPi+LR vaccine	Nobivac & Biocan DHPPi+LR vaccine	Beta--Cov/Vero CC)	Variety PHHV-1	Nobivac & Biocan DHPPi+LR vaccine	Variety (7994)	Variety B/Yamagata

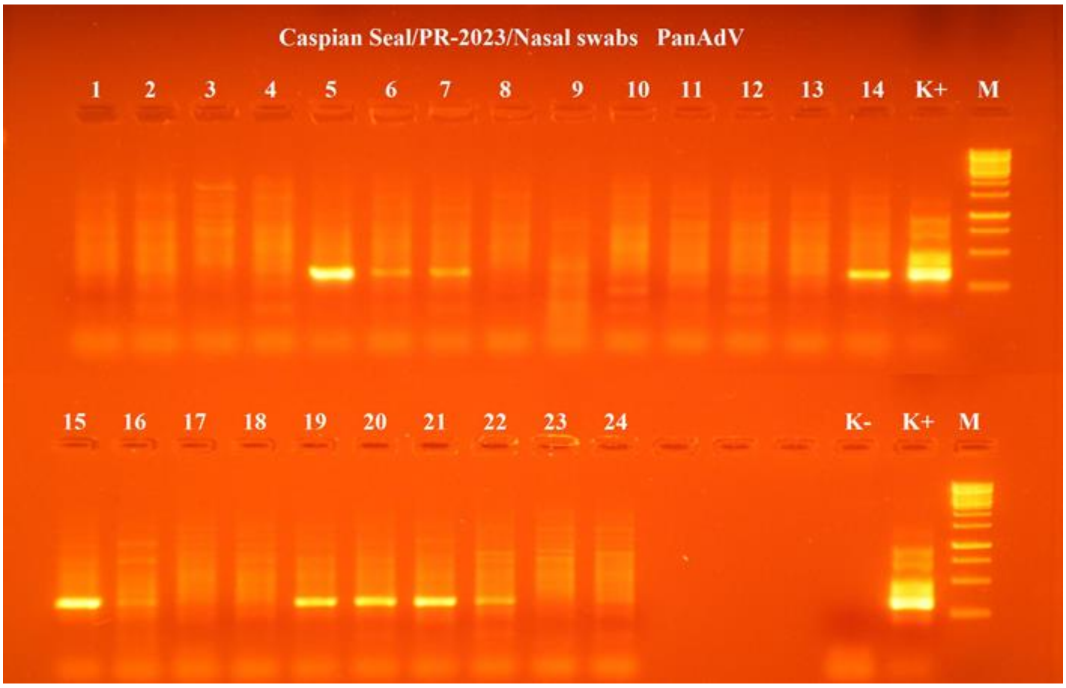


Figure 4.5.2 **Electropherogram of PCR products from biosamples of Caspian seals in 2% agarose gel after amplification with primers to the pol gene of adenoviruses.**

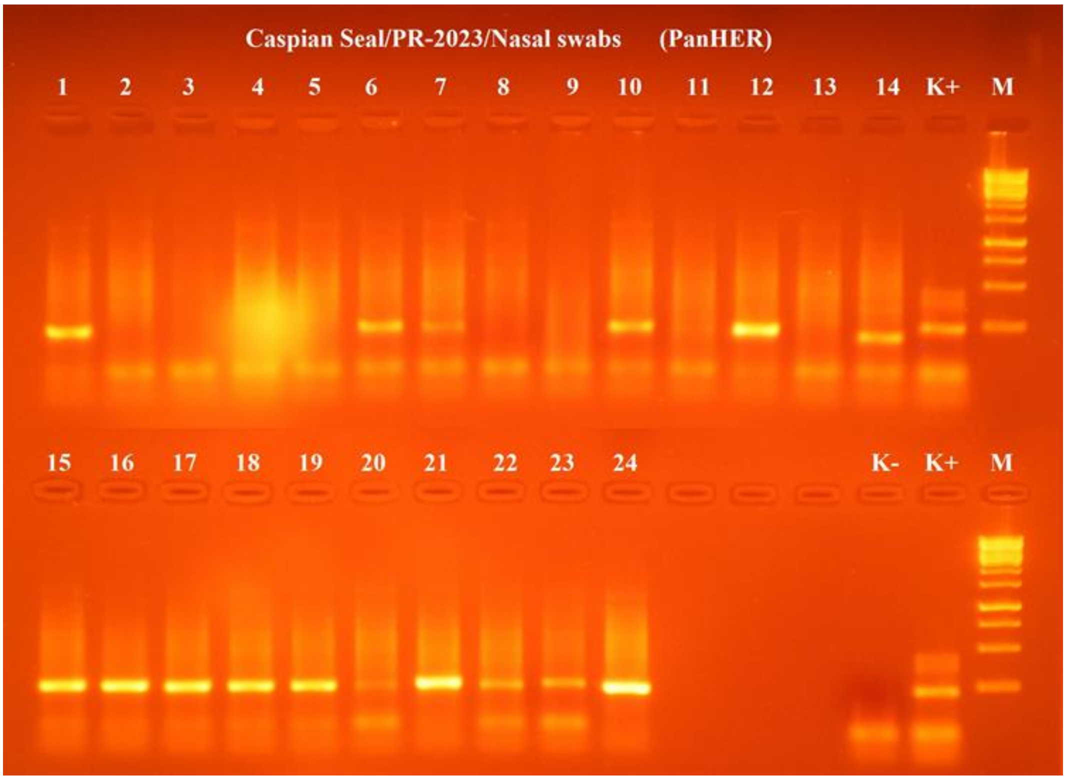


Figure 4.5.3 **Electropherogram of PCR products from Caspian seals in 2% agarose gel after amplification with primers to the Us2 gene of herpesviruses**

The nucleotide sequences of the herpesvirus-positive seal samples showed 89% identity with the DNA polymerase gene fragment of the Hooded seal herpesvirus (Cystophora cristata) isolate FMV04-1493871. Hooded seal herpesvirus Taxonomy ID: 1419110 belongs to the unclassified gammaherpesviruses. These findings contribute to our understanding of the spectrum of herpesviruses in Caspian seals, as previous studies also provided virological and serological evidence of alpha-herpesvirus circulation in the population of these animals [Kydyrmanov A., Karamendin K., etc., 2023; Kydyrmanov A.I., Karamendin K.O., Kasymbekov E.T., 2023].

Phylogenetic analysis of the nucleotide sequence fragment of the Us2 gene of the Caspian seal herpesvirus established that the studied virus belongs to the gammaherpesviruses of seals (Phocine herpesvirus serotype 2, PhHV-1), but sequencing of the complete genome is necessary for a comprehensive characterization of the virus and identification of its relationship with other herpesviruses of pinnipeds (Figure 4.5.5). Analysis of the Caspian seal herpesvirus genome fragment shows its relation to seal herpesviruses of serotype 2, such as gammaherpesviruses of harp seals and ringed seals. Clinical manifestations of infections with gammaherpesviruses in the mentioned pinniped species have not been described.

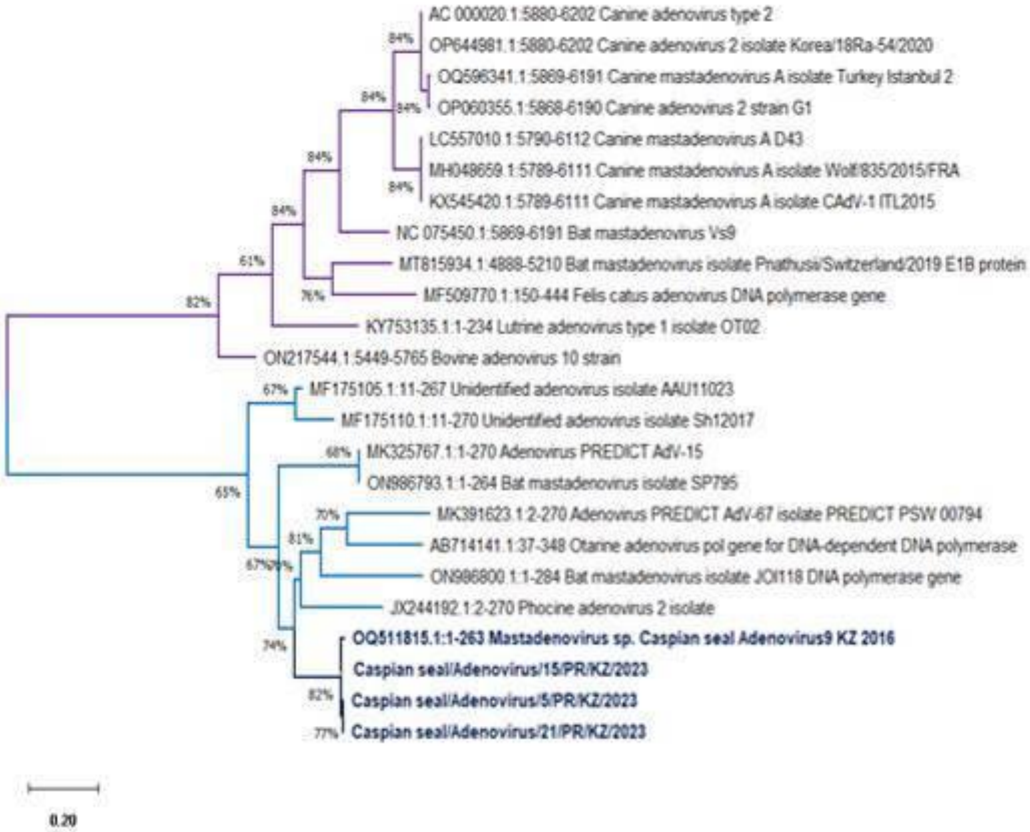


Figure 4.5.4 **Phylogenetic tree of consensus sequences of a fragment of the Caspian seal adenovirus DNA-dependent DNA polymerase gene, consisting of 240 nucleotide sequences**

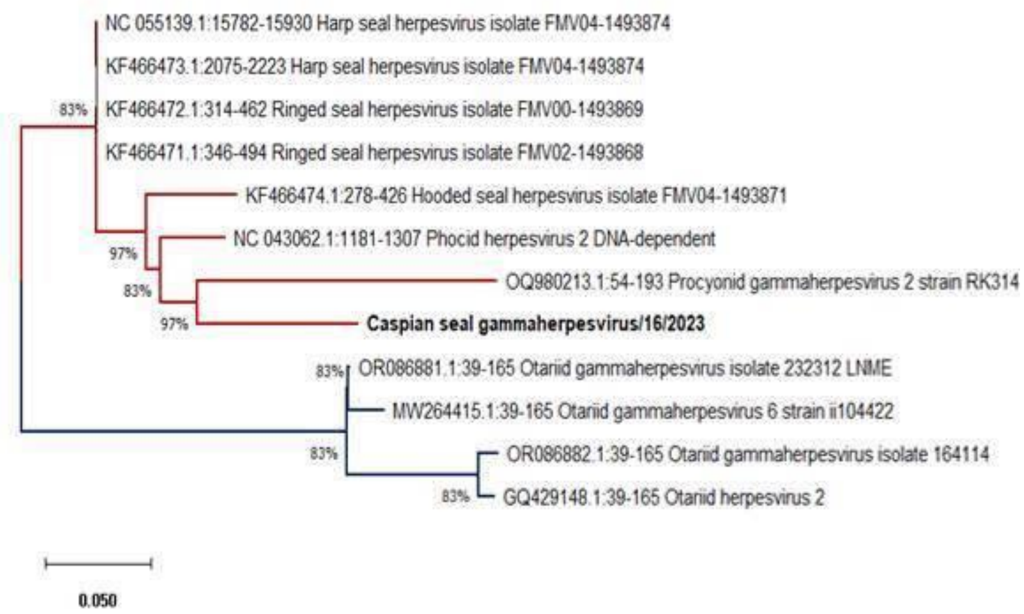


Figure 4.5.5 **Phylogenetic tree of consensus sequences of the DNA-dependent DNA polymerase (DPOL) fragment of herpesvirus, consisting of 175 nucleotides of sequence.**

Analysis of the partial sequence of the Us2 gene suggests that the Caspian seal herpesvirus variant is very similar to the alphaherpesvirus of seals (Phocine herpesvirus serotype 1, PhHV-1), but sequencing of the complete genome is necessary to fully characterize the virus and its relationship to other seal herpesviruses.

Based on the samples studied, the data obtained indicate the presence of mixed respiratory infections among the studied group of animals, caused by adenoviruses and seal herpesviruses serotype 1. The dominant role of viruses in infectious pathology requires additional research.

4.5.1.2. Study of the viral metagenome of the Caspian seal

A total of 20 harbor seals were sampled for analysis and pooled based on sex and age. Eight types of pools were obtained: nasal, oral, urogenital, and rectal flushes from young and adult males, young and adult females, pools of organs from fallen animals, and aborted fetuses. Additionally, lung samples from fallen seals from Dagestan (Russia) were delivered to the laboratory. These samples were used for library preparation and sequenced on Illumina HiSeq4000 from Macrogen, Inc. (Republic of Korea).

A total of approximately 1,250,000 raw sequenced reads per sample were obtained. BLAST searches of the obtained contigs using a threshold value of 10E-5 revealed the presence of viruses belonging to the families Anelloviridae, Caliciviridae, Circoviridae, Coronaviridae, Herpesviridae, Orthomyxoviridae, Papillomaviridae, Parvoviridae, and Picornaviridae (Table 4.5-3). Viral contigs showed a wide range of identity to sequences (71-100%) of the genome fragment of known viruses. It is hypothesized that some of these sequences may be derived from new virus species.

Table 4.5-3 **Number of contigs for families, genera, and species of Caspian seal viruses with maximum coverage, percentage of alignment identity, and maximum contig lengths**

Sex, age of animal / type of sample	Virus family	Virus genus	Variety	Max. coverage	Max. id, %	Max. contig length, bp.	Number of contigs
Adult females /nasal flush	Anelloviridae	Lambdatorquevirus	Torque teno pinniped virus 3	26,012	78,98	2278	6
		Nutorquevirus	Torque teno seal virus 4	66,054	71,48	1366	3
	Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 1	5	100	321	1
	Caliciviridae	Vesivirus	San Miguel sea lion virus 8	20,028	78,11	861	1

Sex, age of animal / type of sample	Virus family	Virus genus	Variety	Max. coverage	Max. id, %	Max. contig length, bp.	Number of contigs
Adult females /oral flush	Papillomaviridae	Betapapillomavirus	Betapapillomavirus 1	47,216	78,79	359	2
		unknown	Leptonychotes weddellii papillomavirus 6	3663	80,95	1342	13
	Herpesviridae	Percavirus	Phocid gammaherpesvirus 3	52	94,73	1667	13
	Anelloviridae	Nutorquevirus	Torque teno seal virus 4	1659,93	82,99	326	1
	Circoviridae	unknown	Circovirus-like genome DCCV-4	7,044	98,73	300	2
Adult females /rectal flush	Parvoviridae	Aquambidensovirus	Asteroid aquambidensovirus 1	5	83,28	798	1
	Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 1	2,006	99,62	484	5
	Anelloviridae	unknown	Seal anellovirus TFFN/USA/2006	1935,90	87,90	2300	1
	Circoviridae	unknown	Circovirus-like genome DCCV-4	4,027	99,15	292	1
Adult females/Urogental flush	Anelloviridae	Nutorquevirus	Torque teno seal virus 4	47,06	82,71	927	7
		unknown	Seal anellovirus TFFN/USA/2006	252,542	84,93	1051	3
		Lambdatorquevirus	Torque teno pinniped virus 3	59,073	79,12	954	1
	Parvoviridae	Chaphamaparvovirus	Rodent chaphamaparvovirus 1	136,947	78,53	3027	24
	Circoviridae	unknown	Circovirus-like genome DCCV-4	52,185	100	422	5
	Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 1	12,064	100	327	1
	Coronaviridae	Betacoronavirus	Severe acute respiratory syndrome-related coronavirus	14,08	100	316	1
Brain	Orthomyxoviridae	Alphainfluenzavirus	Influenza A virus	4,017	93,89	371	1
	Coronaviridae	Betacoronavirus	Severe acute respiratory syndrome-related coronavirus	55,188	99,53	434	1
	Papillomaviridae	Betapapillomavirus	Betapapillomavirus 1	36,073	99,69	637	1
		Gammapapillomavirus	Gammapapillomavirus 1	15,028	100	672	2
Adults of both sexes / lungs	Orthomyxoviridae	Alphainfluenzavirus	Influenza A virus	12,047	89,39	394	1
	Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 1	93,085	100	1236	5
Adults of both sexes / liver, spleen, kidneys	Parvoviridae	Erythroparvovirus	Pinniped erythroparvovirus 1	2,085	92,11	539	3
	Herpesviridae	Rhadinovirus	Bovine gammaherpesvirus 4	2	84,27	349	1
	Circoviridae	unknown	Circovirus-like genome DCCV-4	3,011	99,27	416	1
Adults of both sexes / lymph nodes	Picornaviridae	Hepatovirus	Hepatovirus B	4	92,86	367	1
Aborted fetus / lungs	Circoviridae	unknown	Circovirus-like genome DCCV-4	616,05	100	342	1
Aborted fetus / liver, spleen, kidneys	Parvoviridae	unknown	Psittaciform parvoviridae sp.	1	99.70	593	3

(a) The level of identity was determined based on the reference strain in the RefSeq virus database (NCBI reference sequence database). The analysis was conducted using BLAST X (E-value < 1 × 10 -10).

The nasal flushes of adult animals were dominated by viruses of the family Anelloviridae (varieties of pinniped viruses 3-4), Papillomaviridae (γ-papillomavirus-1), and Caliciviridae (San Miguel sea lion virus) (Figure 4.5.6). Mouth flushes of Caspian seals were characterized by the presence of β-papillomavirus 1, Weddell seal papillomavirus 6, γ-herpesvirus 3, and Circovirus-like genome DCCV-4. Viromes of rectal flushes of adult females revealed contigs of viruses of families Parvoviridae

(Asteroid aquambidensovirus 1) and Anelloviridae (seal anellovirus TFFN/USA/2006). The virome of urogenital flushes of females was characterized by the presence of β-coronavirus.

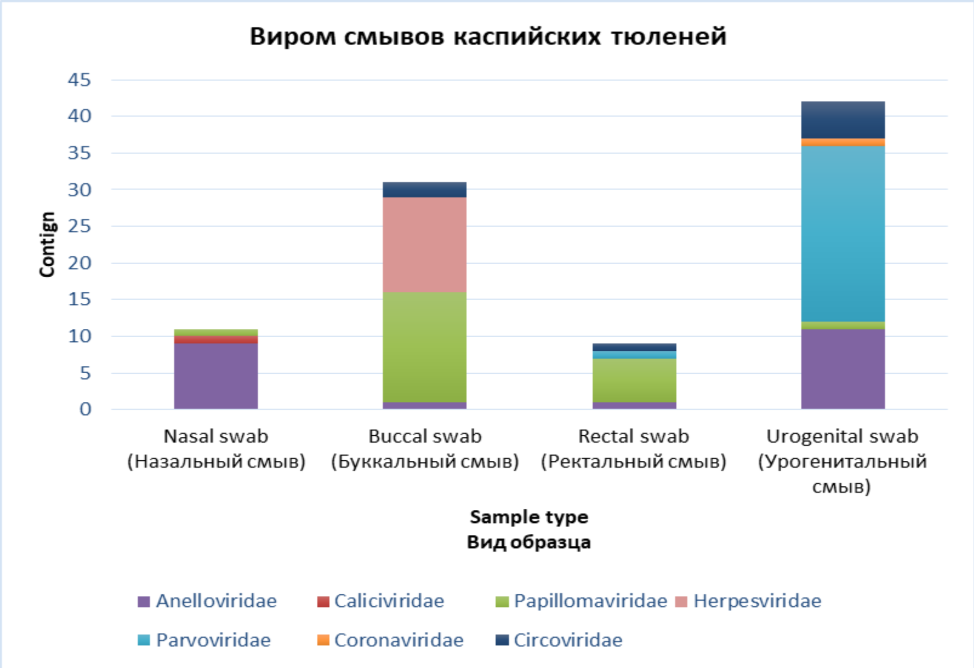


Figure 4.5.6 **Characterization of the virome from oral, nasal, urogenital, and rectal flushes of adult female Caspian seals**

Contigs representing viruses from the families Orthomyxoviridae, Papillomaviridae, and Coronaviridae were detected in the brain tissues of deceased seals (Figure 4.5.7).

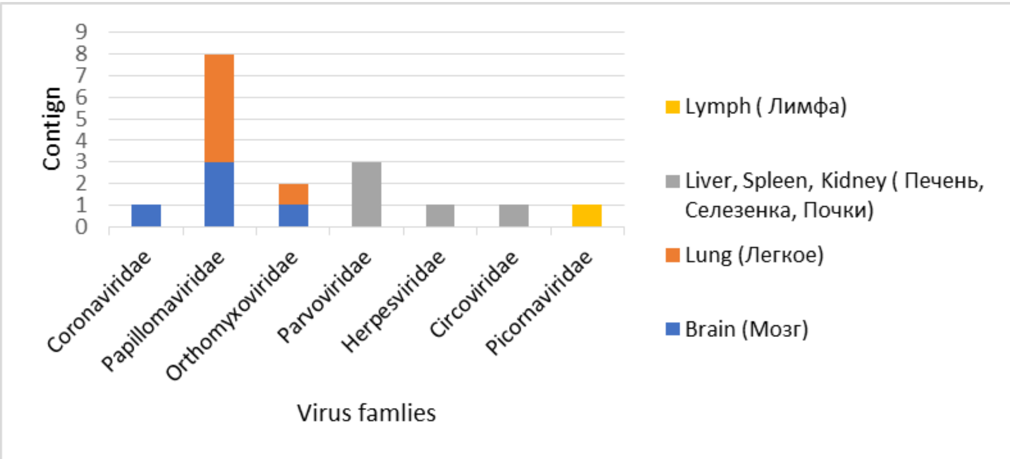


Figure 4.5.7 **Characterization of the virome from internal organs of deceased Caspian seals**

Influenza A virus contigs (family Orthomyxoviridae) and γ-papillomaviruses were identified in the lungs of the seals. Sequences specific to virus families Parvoviridae, Herpesviridae, and Circoviridae were revealed in the pool of internal organs from deceased seals. The viral population in internal organs (liver, spleen, kidneys) included herpes-, parvo-, and circoviruses. Contigs of Hepatovirus B (family Picornaviridae), known as seal phopivirus, were found in the lymph nodes of a deceased seal.

Considering the spatial distribution of viruses, the entire Caspian Sea should be regarded as a single collection site for material, as Caspian seals do not exhibit specific associations with any part of the sea; instead, they move throughout the sea without forming distinct populations. Specific patterns in virus distribution according to sample type can be discerned from the obtained results (Figure 4.5.8).

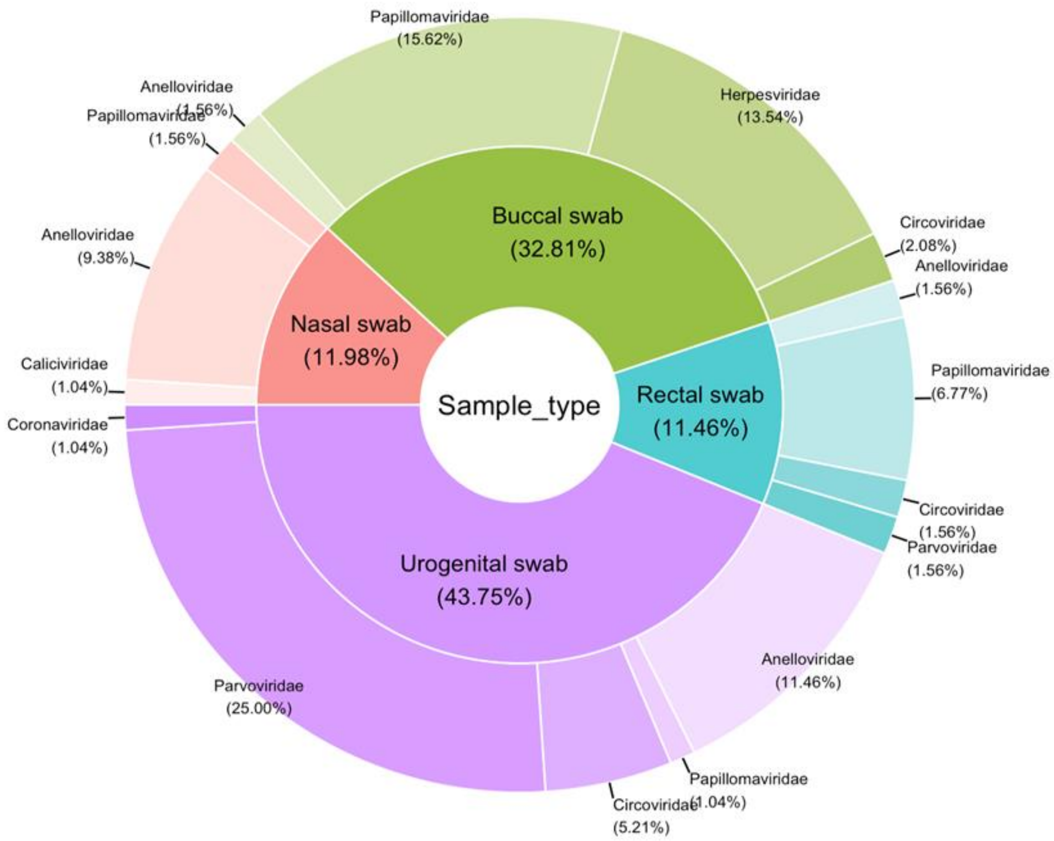


Figure 4.5.8 **Ratio of contigs associated with viruses from different families**

The study revealed no difference in the prevalence of viruses among young or adult animals of both sexes. Below is a description of each virus family found.

The Circoviridae family emerged as the most common virus family in this study. Members of the Circoviridae family are small, unencapsulated, circular single-stranded DNA viruses identified in a variety of mammals, birds, and other organisms worldwide. Phylogenetic analysis showed the closest similarity between the Caspian seal circovirus and the zircon-like virus 2 associated with humpback whale respiratory disease discovered in Australia in 2017. Circoviridae-like viral sequences are widely distributed in the marine environment and may be associated with many aquatic organisms.

The second most abundant family is Parvoviridae, which includes single-stranded linear viruses with single-stranded DNA. In this study, sequences showing 58.8% similarity to canine parvovirus were found in Caspian seals, but we cannot consider them closely related to this pathogenic virus species. Instead, they are related to parvoviruses associated with aquatic ecosystems. Phylogenetic analysis of the partial sequence of the NS2 nonstructural protein showed clustering of Caspian seal parvovirus with densoviruses distantly related to the pathogenic canine parvovirus. Caspian seal densovirus clustered with densoviruses of oysters, clams, and starfish, which are also associated with marine aquaculture.

Herpesviridae is a large family of enveloped viruses with double-stranded DNA. Herpesviruses are frequently reported in marine mammals. Sequences detected in Caspian seals by Us2-like protein are 97% similar to those of harp seal herpesvirus alpha 1, described in Atlantic harbor seals in 1985.

Genomic sequences of two types of herpesviruses have been detected in mouth flushes, such as KRS herpesvirus 6 and Phocid herpesvirus 3 (harp seal herpesvirus).

Papillomaviruses are small enveloped viruses with double-stranded DNA that infect the skin or mucous membranes of various animal species. Among marine mammals, papillomaviruses were identified in harbor porpoises (Phocoena phocoena) in 1999.The Caspian seal sequences are 72.58% similar to the E2 protein previously identified as Weddell seal papillomavirus 6. γ-papillomaviruses were present in samples from both groups of animals. In samples from adult animals, β-papillomaviruses were additionally detected.

Picornaviruses are small, enevloped viruses with positive sense single stranded RNA of the Picornaviridae family found in the Caspian seals samples examined. The sequences of the Caspian seal picornaviruses are 70% similar to the raccoon dog picornavirus isolated in China in 2017-2021.

Caliciviridae is a family of enevloped, single-stranded viruses with positive sense RNA. Marine caliciviruses belong to the genus Vesivirus of the family Caliciviridae. It has been suggested that marine mammals serve as their natural reservoir. The Vesivirus-like sequences found in this study were 71% similar to the San Miguel sea lion virus isolated in the USA. Phylogenetic analysis also confirmed its relatedness to San Miguel sea lion virus and canine vesiviruses. Anelloviruses are enevloped, ring viruses with single-stranded DNA belonging to the family

Anelloviridae. They are very diverse and have not been linked to any disease until now. The sequence of Caspian seal anellovirus was 78% similar to that of harbor seal anellovirus 4. Phylogenetic analysis of the partial nucleotide sequence of ORF1 of Caspian seal anellovirus showed its relatedness to the cluster consisting of seal anelloviruses 4 and 5.

Orthomyxoviruses are enveloped viruses with segmented RNA. In 2009 samples, contigs of Influenza A virus were detected. The virus showed 94-95% similarity in hemagglutinin and nonstructural protein genes to influenza viruses isolated from wild birds between 2006 and 2019. The influenza virus from Caspian seals belonged to the H3 subtype previously described in pinnipeds.

In conclusion, two large groups consisting of different virus families are noted: the first group consists of the families Circoviridae and Parvoviridae, associated with the aquatic ecosystem. The primary hosts of these viruses are various organisms in the marine environment of the Caspian Sea. For example, invertebrates in the case of circoviruses or insects and crustaceans in the case of densoviruses of the Parvoviridae family. It should be noted that this group accounts for 72% of the total Caspian seal virome. They are likely of dietary origin. The second group comprises mammalian viruses: Herpesviridae, Papillomaviridae, Caliciviridae, Anelloviridae, and Orthomyxoviridae. Viruses in this group have the potential to cause various pathologies in mammals or remain asymptomatic. It is still unclear to which group the Picornaviridae-like sequences belong, as the identified contigs are genetically distant from mammalian and aquatic viruses. The similarity of Caspian seal viruses to viruses found in other animals worldwide ranged from 58 to 100%. Given that the habitat of the Caspian seal is confined to the enclosed waters of the Caspian Sea, which has no direct access to the World Ocean, the detection of genetically distant viruses was anticipated.

Caspian seals are regularly found dead, with up to several hundred carcasses washing ashore each year. Unfortunately, in such cases, it is not always possible to determine the primary cause of death, highlighting the need for regular studies on seal viruses.

The studies conducted have some limitations. Firstly, only short viral sequences have been identified, and their phylogenetic position requires further confirmation. Secondly, PCR/OT-PCR confirmation was not carried out in all cases due to a lack of reliable primers. Thirdly, counting viral contigs for prevalence analysis is a common practice, but it serves as an indirect indicator.

4.5.1.3. Characterization of the microbiome of Caspian seal respiratory, gastrointestinal, and urogenital tracts by sequencing the 16S ribosomal RNA gene of microflora.

Bacterial analysis was conducted by sequencing a fragment of the 16S ribosomal RNA of microflora using the Illumina protocol to identify pathogens responsible for bacterial infections in the respiratory and gastrointestinal tracts of Caspian seals. Genospecific primers targeting the 16S V3 and V4 regions were utilized, along with NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1), E7335S (24 reactions) kits. For this purpose, oral, urogenital, and rectal flushes from Caspian seals were grouped by sex and age. The obtained sequencing data were then subjected to bioinformatic analysis using the Geneious 16S Biodiversity Tool program.

In total, taxa representing 14 types of bacteria were identified in the microbiome of Caspian seals, with five types predominating in the dataset: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Fusobacteria (Figure 4.5.9).

Actinobacteria produce a wide range of secondary metabolites, including enzymes, antibiotics, antioxidants, and cytotoxic compounds. The presence of Actinobacteria is a characteristic feature of the microbiome of Caspian seals, and this type is found in all pinniped species.

Taxa belonging to the phylum Bacteroidetes dominated the microbiome of Caspian seals. The abundance of the genus Bacteroides was associated with the carnivorous diet of seals, which is characterized by high levels of proteins, amino acids, and animal fats. By encoding a large number of active carbohydrate enzymes of animal origin, Bacteroides have a high ability to hydrolyze food glycans, a type of polysaccharide derived from proteins. Thus, the high abundance of the genus Bacteroides found in the present study in seals is consistent with their carnivorous habits. The enzymes found in these types of bacteria may help the seal obtain energy from the prey it consumes.

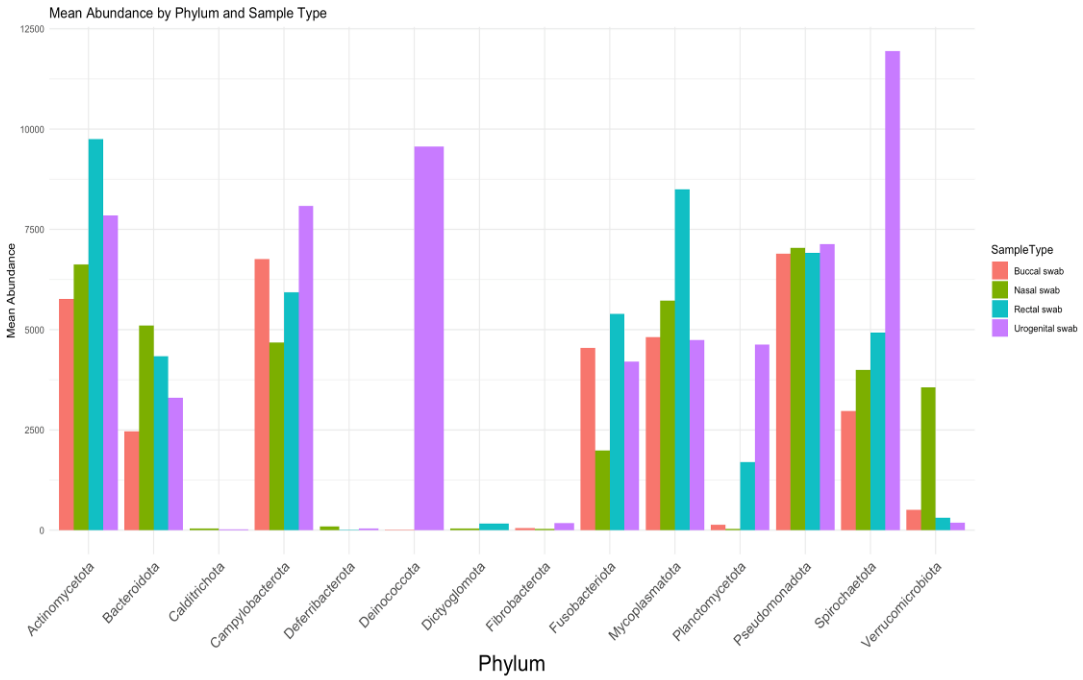


Figure 4.5.9 Primary bacterial taxa present in nasal, oral, rectal, and urogenital swabs obtained from adult female Caspian seals.

Bacteroides metabolize polysaccharides and oligosaccharides, providing nutrition and vitamins to the host and other intestinal inhabitants. Some Bacteroides species can play dual roles, being beneficial in the gut but opportunistic pathogens in other parts of the body, depending on their location within the host.

Firmicutes play a significant role in host immune functions and are commonly found in the digestive tract of many mammalian species, with numerous populations absent in non-host environments.

A higher proportion of Firmicutes compared to Bacteroidetes is associated with greater efficiency in energy extraction from food and fat accumulation. The high ratio of Firmicutes to Bacteroidetes in the rectum of seals is related to their reliance on fat deposition for energy storage and thermoregulation. It can be assumed that intestinal microbes in Caspian seals contribute to efficient fat accumulation necessary for survival by enhancing colon energy availability, similar to observations in humans.

Fusobacteria, renowned for their proteolytic activity in the human gut, were found to be the predominant genus in the gut microbiota of seals, comprising 25%. Marine mammals exhibit a higher abundance of Fusobacteria compared to terrestrial mammals. Members of this genus, such as Fusobacterium, inhabit the oral cavity and intestines of animals. Similar to other marine predators, Fusobacteria were among the most abundant types in the microbiome of Caspian seals.

Proteobacteria were present on various body sites, including the skin, oral cavity, tongue, vaginal tract, and intestine.

The analysis revealed an identical composition of the microbiome in the organs of the deceased animals (Figure 4.5.10).

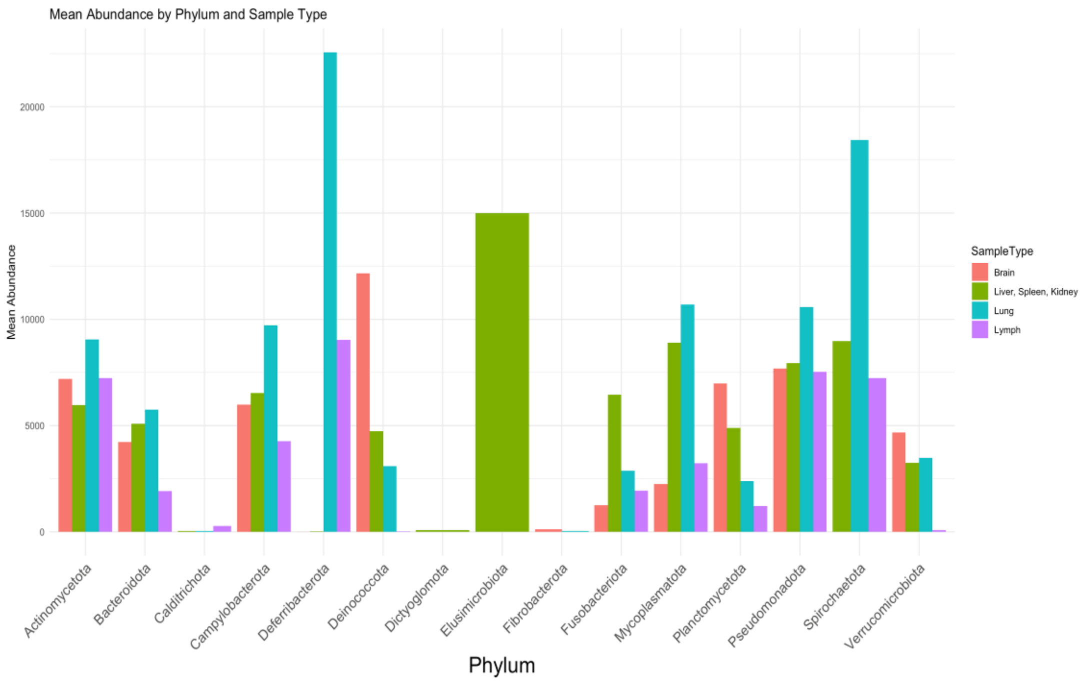


Figure 4.5.10 **Primary bacterial taxa in tissue samples of internal organs from deceased Caspian seals**

During the analysis, it was observed that the composition of the microbiome differs between male and female Caspian seals, with Firmicutes being the predominant type of bacteria (Figure 4.5.11).

In male seals, the microbiome of the oral, nasal, and urogenital regions was primarily composed of Proteobacteria, except for rectal samples. As for the rectal microbiome of Caspian seals, similar to other pinniped species, five main types predominated: Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria, and Actinobacteria. The microbiome of conjunctival flushes from both male and female Caspian seals exhibited low diversity.

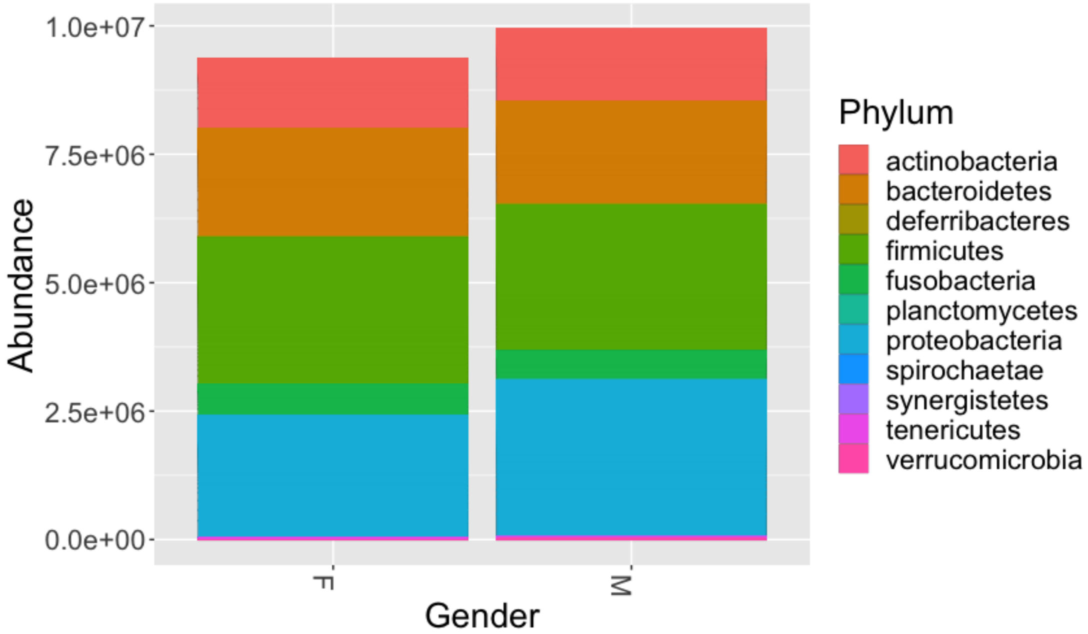


Figure 4.5.11 Microbiome profiles of female and male Caspian seals

The comparative analysis of the Caspian seal microflora revealed similar microbiome profiles in respiratory, alimentary, and urogenital tract flushes of animals collected between 2020 and 2023. Variability in the percentage of bacterial families was observed depending on the type of sample from the Caspian seal. These findings underscore the importance of regular monitoring of the microbiome in Caspian seals to detect the introduction of clinically relevant bacterial pathogens into their population. Overall, the results of this study lay a solid foundation for future research and contribute to the understanding of host-microbe interactions in the Caspian seal population.

4.5.1.4. Serological Study of Circulating Influenza A Viruses and Carnivore Plague Virus (CDV) in the Caspian Seal Population from 2019 to 2023

Thirty-eight serum samples collected from Caspian seals between 2020 and 2023 underwent testing using the IDEXX Influenza A Ab Test Cat.No. 99-53101 ELISA kit, following the protocol outlined by Bodewes et al.

Specific IgG antibodies to the influenza A/NP protein were detected in 25 out of 47 serum samples tested (53.2%). Antibodies against the influenza A virus were found in four samples (30.8%) out of the 13 seal sera collected in 2020. In contrast, 21 out of 25 blood samples collected in November 2022 tested positive for the presence of influenza IgG, representing 84% positivity (as presented in Appendix D, Table D.1).

The high percentage of antibodies against the influenza A virus in serum samples of Caspian seals collected in 2022, compared to those from 2020, demonstrates the high susceptibility of Caspian seals to influenza infection and the active circulation of the pathogen in their population. The obtained serological data complement the results of PCR screening for the presence of the influenza A virus in the samples of seals that washed ashore in the Kazakh and Russian parts of the sea in late 2022 and January 2023, indirectly indicating the involvement of the pathogen in the outbreak of epizootic influenza infection among seals with high lethality.

In enzyme-linked immunosorbent assays, antibodies to the carnivore plague virus (CDV) were not detected in seal sera collected in 2020, whereas they were detected in up to 28% of the 2022 samples. One serum obtained in 2019 and two samples from 2023 were also positive for the presence of antibodies to the carnivore plague virus (as detailed in Appendix D, Table D.2).

In addition to the enzyme immunoassay, sera from seals collected between 2020 and 2022 were subjected to virus neutralization testing to detect antibodies to CDV. The results revealed virus-neutralizing antibodies to CDV in up to 60% (15 out of 25 tested) of the sera collected in 2022. The significant difference in the seropositivity results between the two tests can be attributed to the fact that enzyme immunoassay detects antibodies directed against a single viral antigen (protein), whereas virus neutralization testing involves the interaction between the virus and antibodies targeting different proteins of the pathogen, thereby enhancing the specificity of the reaction.

These findings suggest a recent outbreak of these infections in the population of these animals.

4.5.1.5. Molecular study of Caspian seal parasitofauna

To assess the parasitological status among seals, our objective was to investigate the qualitative and quantitative composition of their parasite fauna, focusing on their role as definitive hosts. Our aim was to characterize the parasites' location within the seals' bodies, their spatial and temporal dynamics, and any specific features related to sex and age that might impact their abundance and development.

We employed a next-generation sequencing method to analyze parasitic worm eggs, aiming to characterize the "helminthobiome" of seals. Specific primers targeting various regions of the genome of parasitic worms belonging to the classes Trematoda, Cestoda, and Nematoda, which included NGS adapters and barcodes, were selected. DNA isolation was conducted using a specialized fecal kit, the PureLink Microbiome Kit (Invitrogen), from the rectal flush samples of 20 Caspian seals.

Caspian seals serve as definitive hosts for Anisakis chupakovi, and their infection rates are crucial for maintaining the epizootic focus in the Caspian Sea.

Based on the analysis of 20 seal fecal samples using the Darling and Scherbovich methods, no helminth eggs, including those of Anisakis chupakovi, were detected. The absence of helminth eggs in the seal feces suggests either a small sample size of the studied material or the absence of infected seals during the research period.

4.5.2. Microbiological studies

Microbiological studies utilized samples from the nasal, rectal, and urogenital microbiota. Initial data on the structure of the Caspian seal mucosal microbiome were acquired in 2019, revealing that the limited sample size, with a diverse sex and age composition, was insufficient for comprehensive characterization. In 2022, the sample size was expanded to include 20 individuals, all of which were adult females. Additionally, in 2023, an extra 10 samples were collected from the available material. In the nasal microbiome analysis, 202,385 high-quality reads were obtained, while 184,187 reads were acquired for the rectal microbiome, and 201,831 reads were gathered for the urogenital microbiome. These reads were clustered and annotated into 262 operational taxonomic units (OTUs).

To characterize the community stability, the following parameters of alpha diversity were calculated: Shannon index, Simpson index, Chao index.

- The Shannon index characterizes biological diversity; it usually ranges from 1.8 to 4.8 for mucosal communities;
- The Simpson index characterizes the community evenness: how evenly the specimens (reads) are distributed among the species. For maximally equalized communities, the Simpson index tends to one;
- The Chao index reflects the number of unique operational-taxonomic units in the microbiome, including the smallest ones.

Healthy sustainable communities usually have high Shannon and Simpson indices. A low Shannon index indicates the vulnerability of the community. A low Simpson index indicates current dysbiosis and possible physiological problems.

Reference data for normal physiological values of alpha diversity parameters are presented in Table 4.5-4.

Table 4.5-4 Alpha diversity parameters of the Caspian seal mucous membrane microbiomes (Mean ± SD)

Index	Mucosa		
	Nasal	Rectal	Urogenital
Chao1	31,3 ± 16,4	42,0 ± 13,8	54,3 ± 22,5
Shannon	2,19 ± 0,53	3,48 ± 0,59	3,74 ± 0,61
Simpson	0,65 ± 0,13	0,85 ± 0,06	0,86 ± 0,06

4.5.2.1. Nasal Microbiome

The microbiome of the Caspian seal nasal mucosa is characterized by the following alpha-diversity parameters:

- The Shannon index is 2.19, which indicates a low diversity of the community, but expected for the nasal mucosa;
- The Chao index is 31.3, which reflects the number of unique operational-taxonomic units in the microbiome;
- The Simpson index is 0.66, which indicates a fairly even distribution of frequencies of microorganisms in the community, without pronounced dominants.

However, specimens No1 and No17 showed a sharply reduced diversity with low evenness.

Taxonomically, at the phylum level, the nasal microbiome of the Caspian seal is dominated by Bacteroidetes, Actinobacteria, and Proteobacteria. The proportion of Firmicutes and Epsilonbacteraeota is at least 1% (Figure 4.5.12).

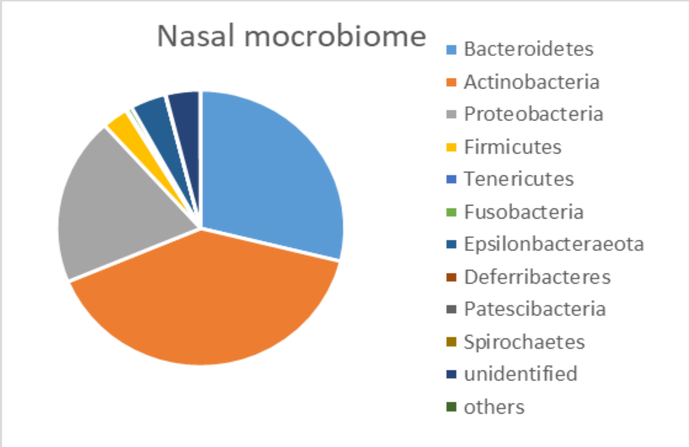


Figure 4.5.1 Microbiome Structure of the Caspian Seal Nasal Mucosa at the Phylum Level

Individual variability at a high taxonomic level was poorly manifested. Two specimens (No1 and No17) were virtually devoid of Proteobacteria (Figure 4.5.13). At the same time, specimen No.4 contained an uncharacteristic predominant species - Epsilonbacteraeota. The presence of Fusobacteria in specimen No. 7 is noteworthy.

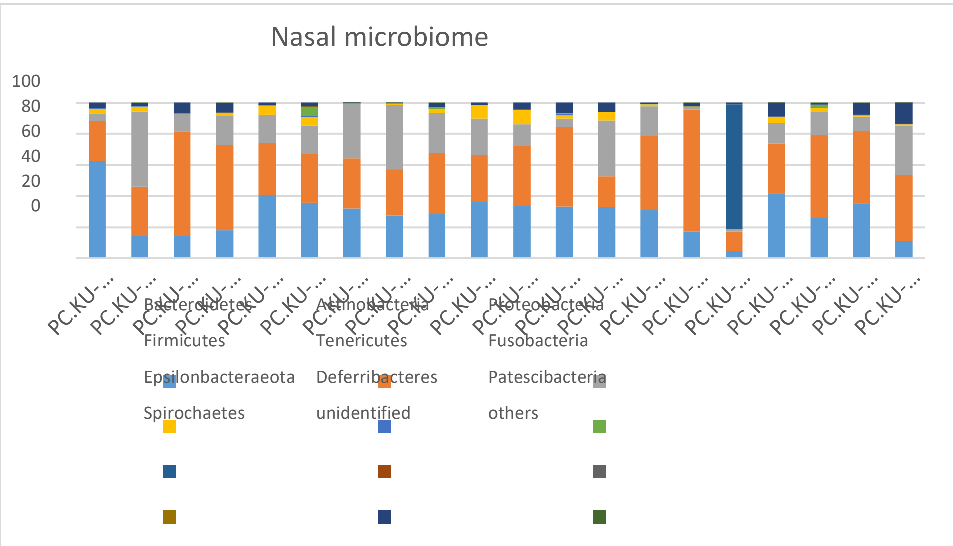


Figure 4.5.2 Individual Structure of the Caspian Seal Microbiome of Nasal Mucosa at the Phylum Level

The predominant families of the nasal microbiome are shown in Figure 4.5.14. At this level, individual differences were more pronounced. Conservative families include only Flavobacteriacea and Corynebacteriaceae. In addition, all specimens except for No.18 had representatives of Moraxellaceae. The proportions of the remaining 15 families varied within in a very wide range.

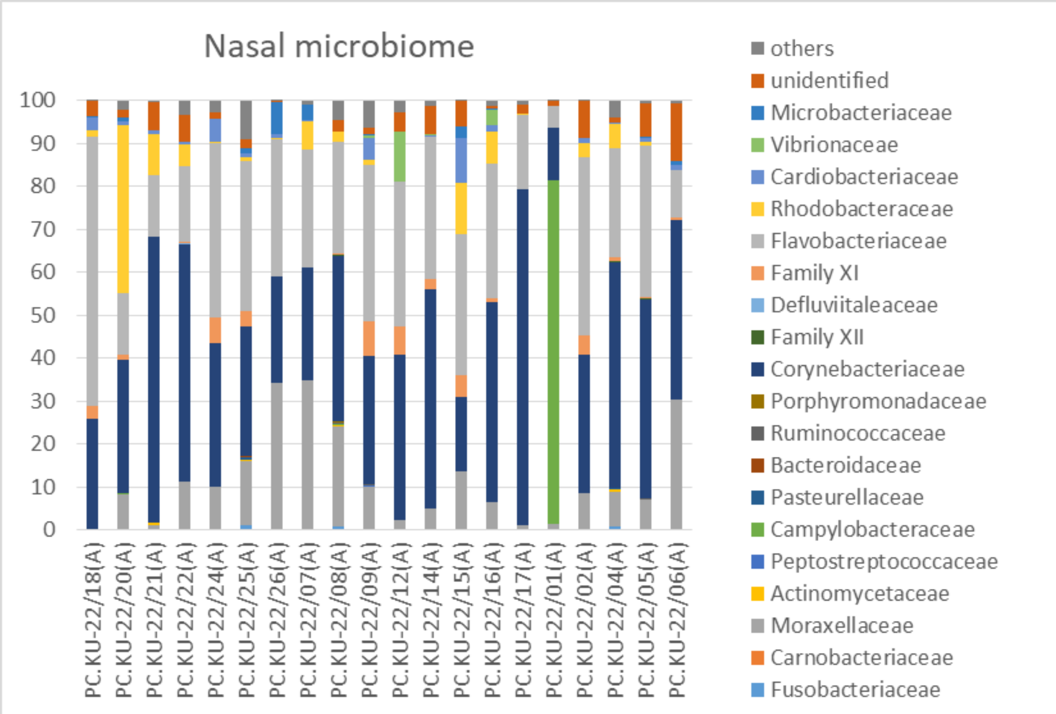


Figure 4.5.3 The Microbiome Structure of the Caspian Seal Nasal Mucosa at the Family Level

The most characteristic microorganisms were identified in the studied microbial communities; they were found in more than 80% of specimens with a proportion of at least 0.1% - the so-called core microbiome (Figure 4.5.15). For the nasal mucosa these are:

Corynebacterium (phocae). Similar nucleotide sequences were previously recorded only in other seals: Arctocephalus gazella (MH728200.1) and Phoca vitulina (NR_026379.1) and were not found anywhere else.

Coenonia (anatine). Similar sequences were recorded in Arctocephalus gazella (MH728227.1), as well as in nasal mucous membranes of ducks and geese that died from respiratory infections.

Arcanobacterium (phocae). Similar sequences were recorded both in other seals and in other carnivorous and herbivorous mammals.

Moraxella sp. Non-specific symbiont of mucous mammals.

Psychrobacter sp. was recorded in all specimens in small numbers. I was previously recorded in Arctocephalus gazella (MH728394.1) and a wide variety of other substrates.

Five specimens Nos. 12, 18, 23, 24, 25 were found to have a microorganism of the presumably Thioclava genus (Rhodobacteraceae family). Similar sequences with a degree of similarity above 97% have not previously been entered into databases.

In general, about 40% of microorganisms are unique for the nasal mucosa of the Caspian seal; about 30% of microorganisms and those from other loci were previously recorded in seals, and another 30% were non-specific microbes.

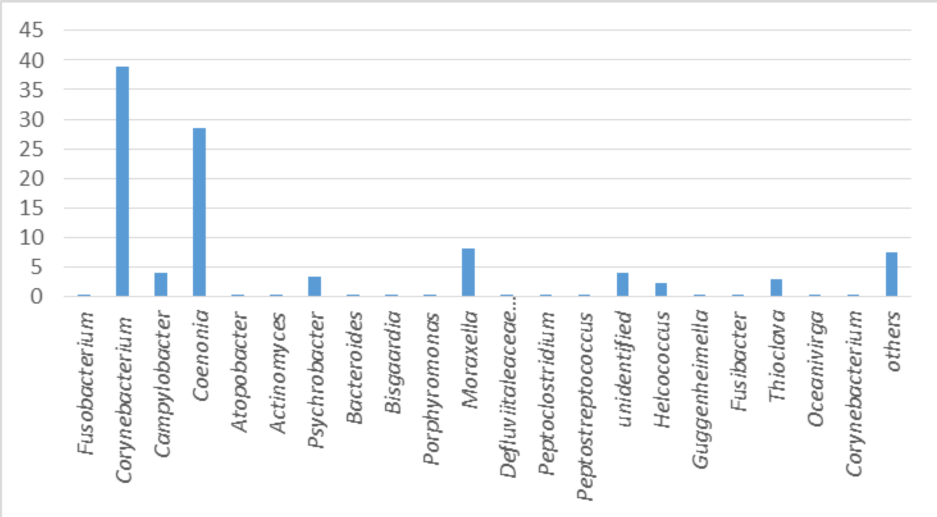


Figure 4.5.4 **Predominant Genera of Microorganisms in the Nasal Mucosa of the Caspian Seal**

4.5.2.2. Rectal Microbiome

The postintestinal microbiome is characterized by the following alpha-diversity parameters:

- Shannon index - 3.45;
- Chao index - 40.7;
- Simpson index - 0.85.

Decreased diversity values (with moderate evenness) were observed in specimens No2 and No12. The predominant phyla include: Bacteroidetes, Actinobacteria, Proteobacteria, Firmicutes, Fusobacteria, Epsilonbacteraeota. Small numbers of Patescibacteria and Spirochaetes are also observed. (Figure 4.5.16).

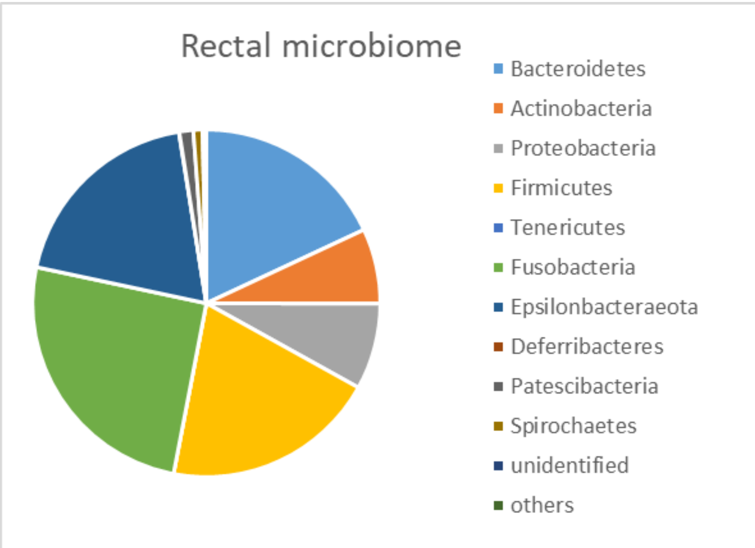


Figure 4.5.5 **The Microbiome Structure of the Rectal Mucosa of the Caspian Seal at the Level of Phyla**

The highly conserved phyla included: Bacteroidetes, Firmicutes, Fusobacteria, Epsilonbacteraeota, Actinobacteria, Proteobacteria (Figure 4.5.17). Spirochaetes were recorded in specimens Nos. 22, 8, 15, while Patescibacteria - in specimens Nos. 15 and 16.

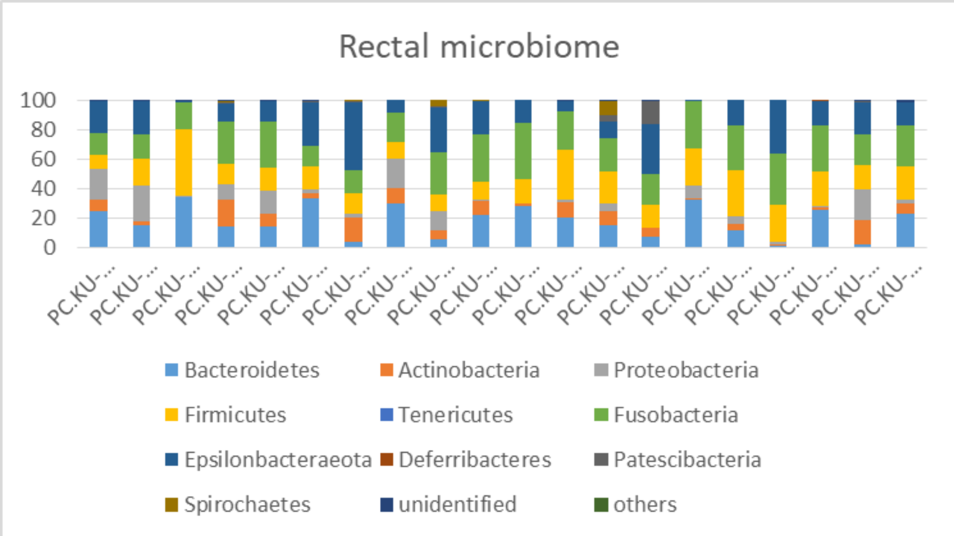


Figure 4.5.6 Individual Structure of the Microbiome of the Caspian Seal Rectal Mucosa at the Phylum Level

Fusobacteriaceae and Campylobacteriaceae were recorded in all specimens. The specimens mostly belonged to the families of Actinomycetaceae, Peptostreptococcaceae, Pasteurellaceae, Clostridiales fam. XII, Defluviitaleaceae (Figure 4.5.18).

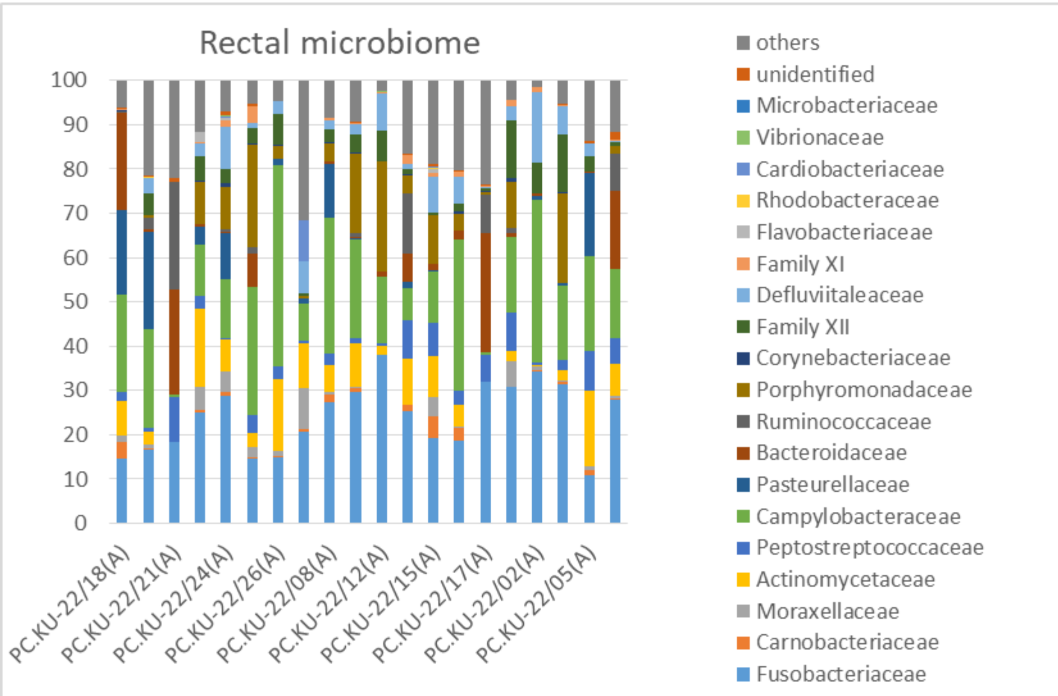


Figure 4.5.7 Individual Structure of the Microbiome of the Caspian Seal Rectal Mucosa at the Family Level

Bacteria characteristic for the rectal mucosa include:

Fusobacterium sp. Similar consequences were previously recorded in Zalophus californianus (JQ204533.1), Arctocephalus gazelle (MH728206.1), Tursiops truncatus (JQ210313.1), and other animals.

Campylobacter sp. Similar consequences were prevsiouly recorded in seals Phoca vitulina (CP053841.1), Zalophus californianus (JQ207528.1), Leptonychotes weddellii (KM100397.1) only.

Porphyromonas sp. Similar consequences were previously recorded only in seals Zalophus californianus (JQ205526.1) and Arctocephalus gazella (MH728397.1)

Actinomyces (marimammalium). Similar consequences were recorded in seals and other carnivorous and plant-eating mammals.

Guggenheimella sp. Similar consequences were previously recorded only in Tursiops truncatus (JQ208973.1).

Psychrobacter sp. was recorded in all specimens in low numbers. It was previously recorded in Arctocephalus gazella (MH728394.1), and in various substrates.

It should be noted that Arcanobacterium, which is not shown in the diagram because it was not included in the list of typical genera, occurs in three specimens with a very high proportion (more than 20%).

The Bergeyella genus, which dominated in some specimens, was also not included in the list of typical specimens. Its sequence has been previously found only in marine mammals, including Neomonachus schauinslandi (GU196264).

In general, about 50% of microorganisms are unique for the rectal mucosa of the Caspian seal; about 50% were previously recorded in seals, as well as in other loci. Eurybiont microorganisms on the rectal mucosa are observed in exceptional cases.

4.5.2.3. Urogenital Microbiome

The urogenital mucosa microbiome is characterized by the following alpha diversity parameters:

- Shannon index: 3.74;
- Chao index: 54.2;
- Simpson index – 0.86.

A pronounced decrease in diversity and a sharp decrease in evenness is observed in specimen No.8. The microbiome has a high similarity to the rectal microbiome and includes: Bacteroidetes, Actinobacteria, Proteobacteria, Firmicutes, Fusobacteria, Epsilonbacteraeota (Figure 4.5.19).

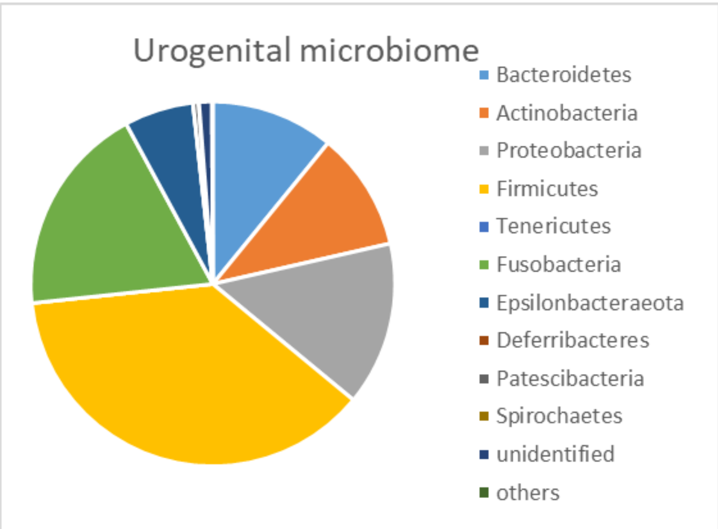


Figure 4.5.8 The Structure of the Microbiome of the Caspian Seal Urogenital Mucosa at the Level of Phyla

Two specimens appeared to have no Bacteroidetes (No.5 and No.21), one specimen (No.21) lacked both Bacteroidetes and Proteobacteria, and No.25 lacked only Proteobacteria (Figure 4.5.20). Interestingly, the rectal microbiome of specimens No.21 and No.5 also lacked Bacteroidetes and

Proteobacteria respectively. The rectal microbiome of specimens No.15 and No.16 appeared to contain Spirochaetes and Patescibacteria respectively.

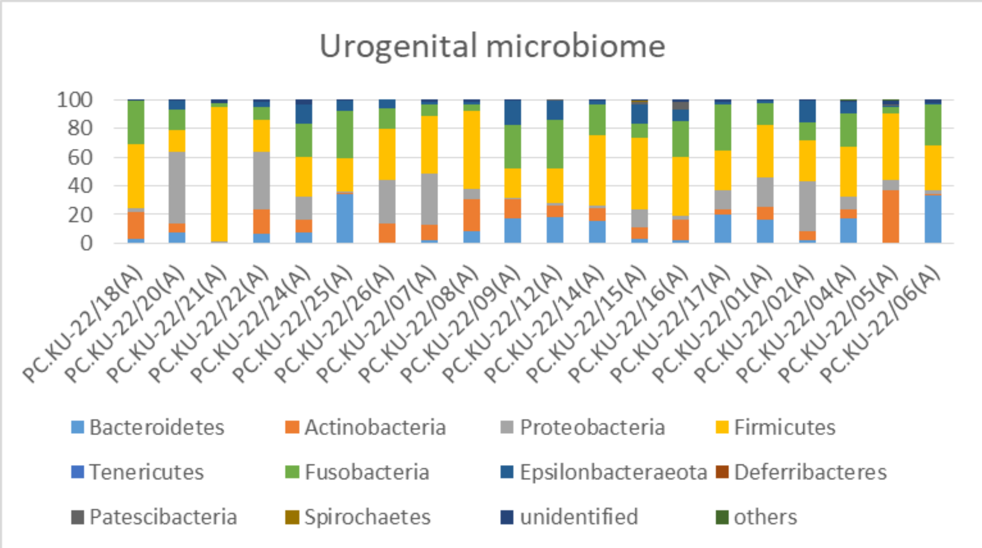


Figure 4.5.9 Individual Structure of the Microbiome of the Caspian Seal Urogenital Mucosa at the Level of Phyla

Although there were no noticeable inter-individual differences at the phylum level, there was great heterogeneity of the families (Figure 4.5.21). Fusobacteriaceae was the only family, whose representatives dominated in all specimens, except for No. 6. Campylobacteriaceae was found in all specimens except for No.18.

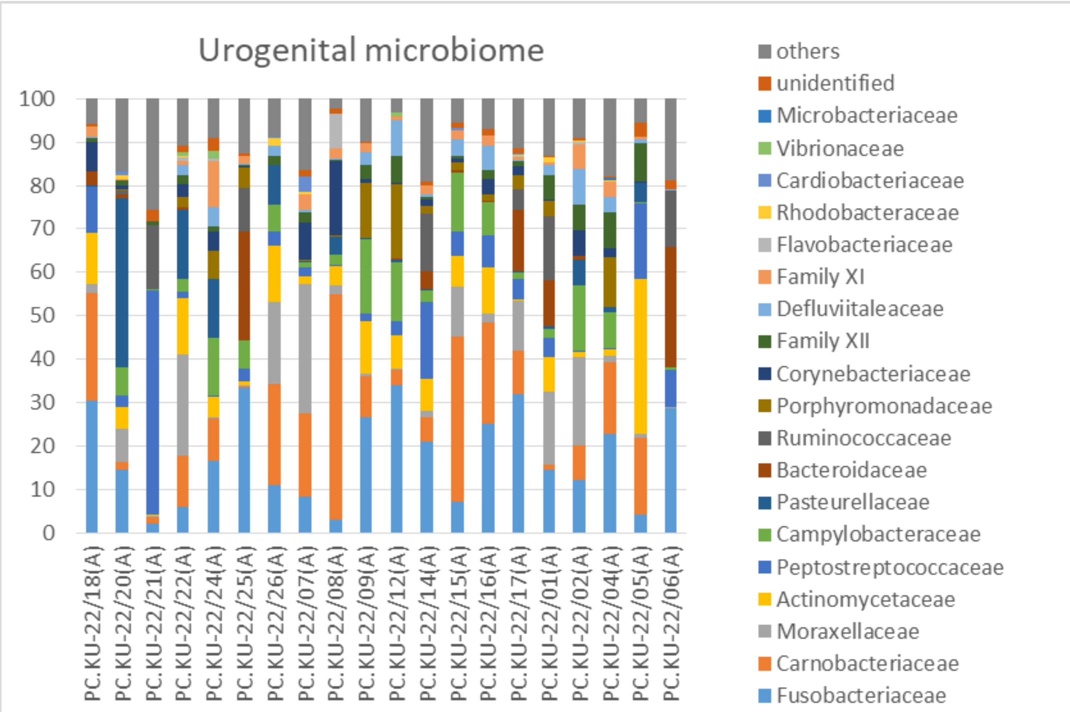


Figure 4.5.10 Individual Structure of the Microbiome of the Caspian Seal Urogenital Mucosa at the Family Level

Almost all microbes characteristic of the rectal mucosa were found in the urogenital mucosa. (Figure 4.5.22): Fusobacterium sp., Campylobacter sp., Actinomyces (marimammalium), Porphyromonas sp., Psychrobacter sp. and:

Atopobacter (phocae). Similar consequences were recorded in Phoca vitulina (NR_119263.1), Leptonychotes weddellii (KM100404.1), Arctocephalus gazella (MH728078.1), and other substrates.

Lachnospiraceae sp. Similar consequences were recorded in Arctocephalus gazella (MH728193.1) and other animals.

Bisgaardia (hudsonensis/ miroungae). A unique situation when the nucleotide sequence was recorded in most of the studied seals: Pusa hispida (CP016605.1), Phoca vitulina (HM626620.1, NR_117488.1), Mirounga angustirostris (KF871287.1), Arctocephalus gazella (MH728268.1), Zalophus californianus (JQ204979.1) and it has not been found anywhere else.

Corynebacterium (caspium), previously described specifically for the Caspian seal, was found only in 80% of the examined specimens. Moreover, a similar sequence was not found in other seals, but it was found in humans and rodents. In addition, a member of the genus Treponema was found in three individuals (No.8, 15, 22), present in both the rectal and urogenital microbiomes. This strain bears a distant resemblance to one isolated from Zalophus californianus (JQ205096.1, 97.2% similarity). However, this sequence has not been encountered elsewhere in international databases.

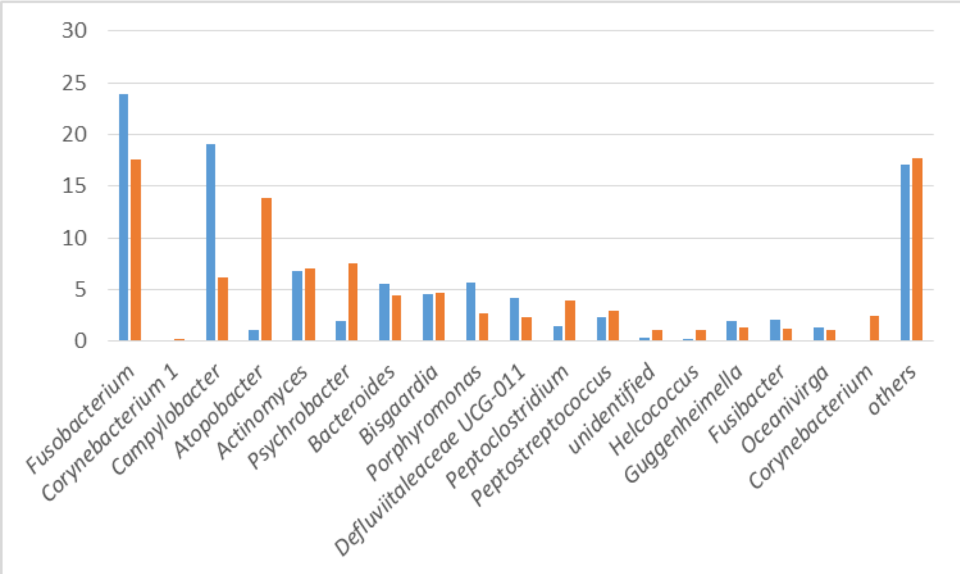


Figure 4.5.11 **Predominant Genera of Rectal (Blue) and Urogenital (Orange) Mucosal Microorganisms of the Caspian Seal**

4.5.2.4. Functional Capabilities of the Caspian Seal

Through bioinformatic analysis of the 16S rRNA structure of the microbiome using the PICRUST algorithm based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, data on the enzymatic capabilities of the community were obtained. Although these are indirect data, the model's accuracy, as used, is estimated to be high (Langille et al., 2013). The analysis revealed that the communities of all three mucosal microbiomes possess similar capacities for amino acid, carbohydrate, and lipid metabolism (refer to Figure 3.25). While the rectal and vaginal microbiomes did not differ significantly in enzyme representation, the nasal microbiome outperformed both the rectal and vaginal microbiomes in enzyme representation for fatty acid (Mann-Whitney test, p= 0.0000001) and amino acid (Mann-Whitney test, p= 0.000001) metabolism. Principal component analysis (refer to Figure 3.26) showed that the rectal and vaginal microbiomes ranked closely in terms of total functionality (PERMANOVA, p>0.05), whereas the nasal microbiome significantly differed (PERMANOVA, p=0.0012).

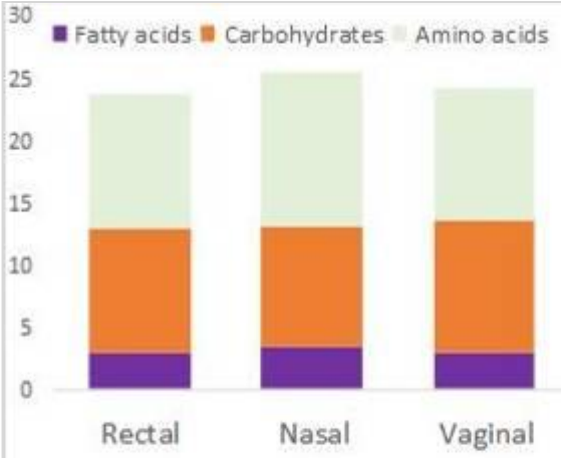


Figure 4.5.12 **Representation of fatty acid, carbohydrate, and amino acid catabolic enzymes on the rectal, nasal, and vaginal mucosa of Caspian seals by PICRUST analysis**

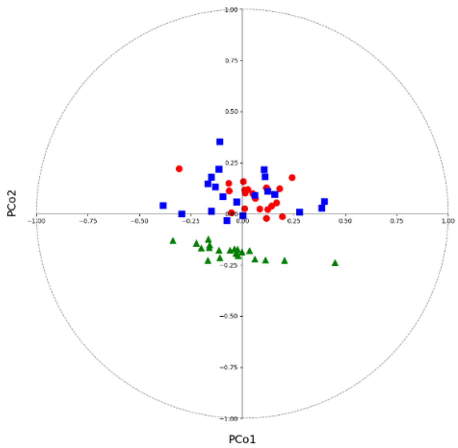


Figure 4.5.24 **Demonstration of similarities in functionality among the rectal microbiome (red markers), vaginal microbiome (blue markers), and nasal microbiome (green markers) using the principal component method.**

Despite the quantitative similarity in the representation of carbohydrate-metabolizing enzymes, fundamental functional differences were observed among the microbiomes of different mucous membranes. When enzymes were grouped by metabolic pathways, it became evident that the rectal and vaginal microbiomes formed a single pattern, while the nasal microbiome exhibited sharp differences from them.

The nasal microbiome demonstrated a higher representation of enzymes involved in the oxidative pathway for carbohydrate and fat degradation. In contrast, the rectal and vaginal microbiomes were characterized by anaerobic degradation of carbohydrates, limited involvement of fats, more intensive fixation of carbon dioxide, and neoglucogenesis. Notably, enzymes involved in oxygen catabolism of carbohydrates were more strongly represented in the nasal microbiome, including pyruvate dehydrogenase [EC:2.3.1.12] by a factor of two and D-lactate dehydrogenase (cytochrome) [EC:1.1.2.4] by a factor of three. Furthermore, the activity of citrate cycle enzymes was more than three times higher in the nasal microbiome, including 2-oxoglutarate dehydrogenase [EC:1.2.4.2], isocitrate lyase [EC:4.1.3.1], 2-oxoglutarate dehydrogenase [EC:2.3.1.1.61], succinyl-CoA:acetate CoA-transferase [EC:2.8.3.18], succinate dehydrogenase / fumarate reductase [EC:1.3.5.1 1.3.5. 4], and enzymes related to the methylcitrate cycle: methylisocitrate lyase [EC:4.1.3.3.30], 2-methylcitrate synthase [EC:2.3.3.5], and 2-methylcitrate dehydratase [EC:4.2.1.79]. Additionally, enzymes associated with the oxidative metabolism of fats were more abundant in the nasal microbiome. Specifically, the representation of 2-methylaconitate isomerase (fat degradation) and 3-hydroxyacyl-CoA dehydrogenase [EC:1.1.1.35] was 10-fold higher, while the representation of pyruvate dehydrogenase (quinone) [EC:1.2.5.1] and acetoacetyl-CoA reductase [EC:1.1.1.36] was 3-fold higher.

On the other hand, enzymes involved in oxygen-free catabolism are more prominently represented in the rectal and vaginal microbiomes. For instance, 6-phospho-beta-glucosidase [EC:3.2.1.86] is 10 times more abundant, 4-alpha-glucanotransferase [EC:2.4.1.25] is twice as prevalent, pyruvate orthophosphate dikinase [EC:2.7.9.1] is 10 times more abundant, and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase [EC:5.4.2.12] is over 2 times more abundant. Additionally, enzymes related to the pentose phosphate pathway, a variant of oxygen-free metabolism of hexoses, are notably well represented. For example, 2-dehydro-3-deoxygluconokinase [EC:2. 7.1.1.45] is 2.5 times more abundant, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase [EC:4.1.2.9 4.1.2.22] is 10 times more abundant, and diphosphate-dependent phosphofructokinase [EC:2.7.1.90] is 20 times more abundant. Also, in the rectal and vaginal microbiomes, enzymes responsible for subsequent steps of anaerobic metabolism, including fermentation, are more strongly represented: butanol dehydrogenase [EC:1.1.1.1.-] less than 2-fold, formate C-acetyltransferase [EC:2.3.1.54] more than 3-fold. They are also characterized by significantly more active carbon dioxide fixation: phosphoenolpyruvate carboxylase [EC:4.1.1.1.31] 5-fold, fumarate hydratase subunit alpha [EC:4.2.1.2], pyruvate carboxylase subunit B [EC:6.4.1.1], and 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha [EC:1.2.7.3, 1.2.7.11] 10-fold. Neoglucogenesis is orders of magnitude stronger (100-fold): fructose-1,6-bisphosphatase III [EC:3.1.3.11].

A comparison of functional enzyme patterns (Figure 4.5.25) revealed that the infectious capacity of the nasal microbiome was lower than that of the rectal and vaginal microbiomes (Mann-Whitney test, p=0.000009). The presence of infectious capacity suggests that a portion of the microbiome comprises facultative pathogens. In other words, when the body's resistance is weakened (due to factors like starvation, stress, or injury), the normal microbiome can instigate a pathological process, even without the presence of a specialized external pathogen. However, the nasal mucosa is comparatively safer in this regard.

In contrast, the nasal community exhibited a higher ability to form biofilms (Mann-Whitney test, p= 0.000007), a significant indicator for the infectious process. This suggests that nasal pathogens can withstand secreted antibodies and other immunity factors for longer durations, and they may also resist antibiotic therapy more effectively. Anticipated antibiotic resistance was similar across all three mucosae, but slightly lower for the rectal mucosa (Mann-Whitney test, p=0.001413). The presence of low antibiotic resistance, regardless of mucosal localization, is a common occurrence. This is a positive sign indicating that animals are not affected by agricultural residues, which often contain high levels of vancomycin.

Furthermore, the notable development of a defense system against aromatic series xenobiotics is observed across all mucous membranes, with a higher capability noted in the nasal community (Mann-Whitney test, p=0.0000001). This indirectly suggests the presence of anthropogenic pollutants from the aromatic series in the water, to which the nasal microbiome primarily responds. It is probable that xenobiotics are metabolized by the host organism before reaching the rectal and vaginal mucosa. An important distinction between the rectal microbiome and the others is its significantly greater ability to recycle nitrogen, attributed to urease activity (Mann-Whitney test, p=0.002). This function of the microbiome has a profound physiological impact on herbivore metabolism, allowing them to consume nitrogen-poor foods. The presence of this pathway in the seal's microbiome, despite being a carnivore that does not require recycling, suggests that not all microbiome abilities should be automatically considered adaptive to the host.

Interestingly, the capacity for vitamin synthesis was maximized in the nasal microbiome but not in the rectal microbiome (Mann-Whitney test, p= 0.00007). This finding suggests that this property of the microbiome is not significant for the seal, despite being crucial for vitamin provision in many other animals.

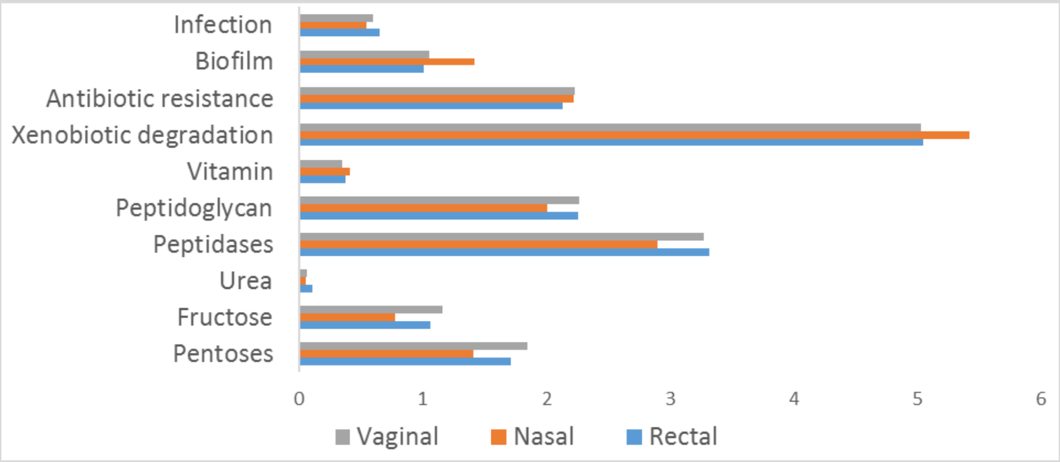


Figure 4.5.13 Comparison of the functional enzyme patterns of the rectal, nasal, and vaginal microbiomes of Caspian seals according to KEGG analysis.

In all three microbiomes, significant amounts of hydrolases that facilitate the breakdown of plant and animal fiber were absent: cellulases, cellobiose hydrolases, laccases, pectinases, and chitinases. The exception was peptidases, which were present in the microbiomes of all mucous membranes, with the highest representation on vaginal mucosa and the lowest on nasal mucosa (Mann-Whitney test, p= 0.00000013). This suggests that the microbiome of seals is not involved in the digestion of complex foods such as algae and chitin shells.

4.5.3. Toxicity Studies

4.5.3.1. Mercury Content in Fur

The mean mercury content in the fur of Caspian seals captured in the fall of 2023 was 2643±1708 µg/kg (n = 24), with a minimum concentration of 754 µg/kg and a maximum concentration of 7052 µg/kg. The frequency distribution of mercury concentrations in the study sample is presented in Table 4.5-5.

Table 4.5-2 Frequency Distribution of Mercury Concentrations in Wool of Examined Caspian Seal Specimens

Hg in wll, µg/kg	Frequency of occurrence, number of specimens	Cumulative frequency of occurrence, number of specimens	Percentage of the total	Cumulative percentage
0–1000	4	4	17	17
1000–2000	6	10	25	42
2000–3000	6	16	25	67
3000–4000	4	20	17	84
4000–5000	1	21	4	88
5000–6000	2	23	8	96
6000–7000	0	23	0	95.8
7000–8000	1	24	4	100

The mean mercury concentration in the fur of females and males was 2921±1742 (n = 20) and 1250±163 (n = 4), respectively (Figure 4.5.26). Despite the difference in mean values, no statistically significant differences in Hg content in fur between seals of different sexes were found (M-W: U = 16, Z = 1.8, p = 0.069).

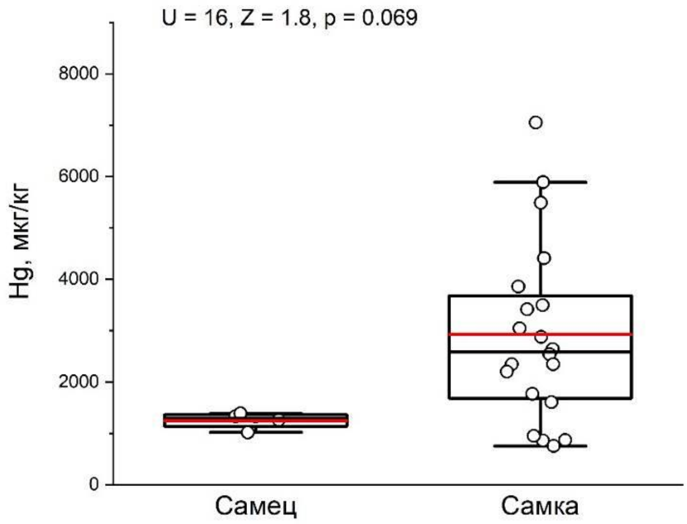


Figure 4.5.26 Content of Mercury in the Fur of the Studied Caspian Seal Males and Females

The mercury content in fur within the study sample showed a positive correlation with age, body weight, and body length, with correlation coefficients (RS) of 0.74, 0.74, and 0.78, respectively ($p < 0.05$) (Figures 4.5.27-4.5.28).

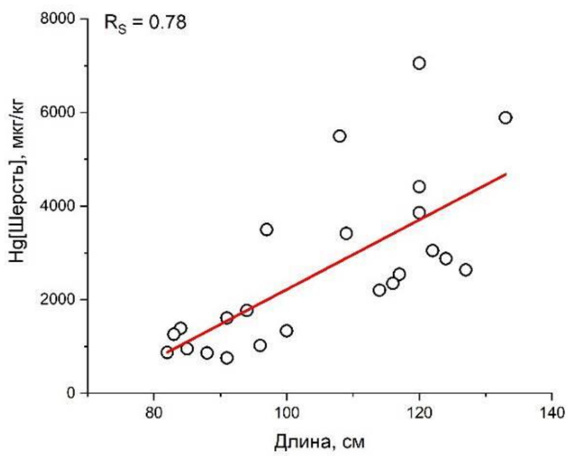


Figure 4.5.147 Content of Mercury in the Fur of the Studied Caspian Seal Specimens Depending on Body Length

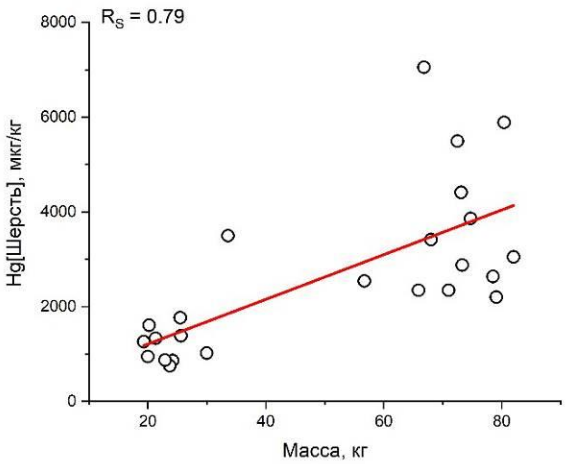


Figure 4.5.15 Relationship between mercury concentrations in hair and the body weight of Caspian seals

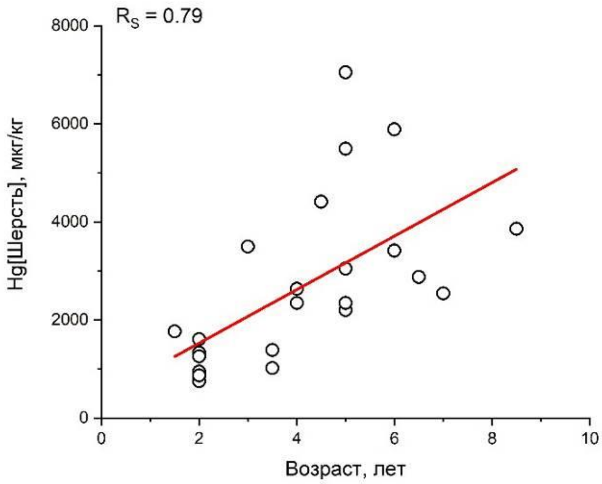


Figure 4.5.16 Relationship between mercury concentrations in hair and the age of Caspian seals

4.5.3.2. Mercury content in vibrissae

Mercury content in vibrissae averaged 3394±1129 μg/kg, with minimum and maximum concentrations of 1734 and 5908 μg/kg, respectively. The most frequent mercury concentrations fell within the range of 2000-4000 μg/kg, occurring in 16 individuals (66% of the total number of spaimens (Table 4.5-6).

Table 4.5-3 Frequency Distribution of Mercury Concentrations in Vibrissae of The Studied Caspian Seal Specimens

Hg in vibrissae, μ/kg	Frequency of occurrence, number of specimens	Cumulative frequency of occurrence, number of specimens	Percentage of the total	Cumulative percentage
0–1000	0	0	0	0
1000–2000	2	2	8	8
2000–3000	7	9	29	37
3000–4000	9	18	37	74
4000–5000	2	20	8	82
5000–6000	4	24	17	100

Mean mercury concentrations in the vibrissae of females and males were 3550±1157 (n = 20) and 2614±573 (n = 4), respectively. As with hair, no statistically significant differences in mercury concentrations in vibrissae were found between seals of different sexes (M-W: $U = 20$, $Z = 1.5$, $p = 0.135$) (Figure 4.5.30).

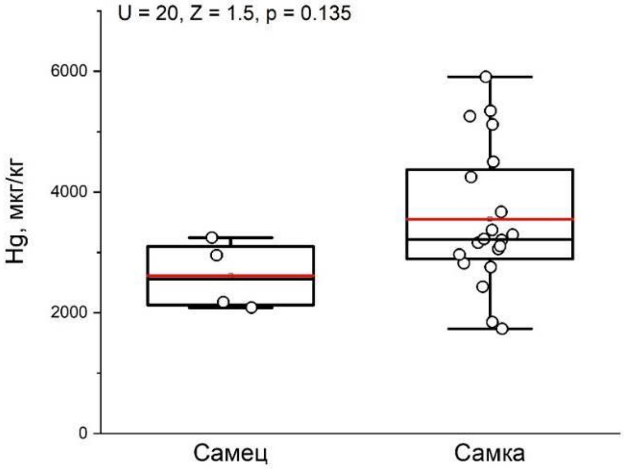


Figure 4.5.17 Comparative Mercury Content in Vibrissae of Male and Female Caspian Seals

Mercury content in vibrissae is statistically significantly higher than in hair (M-W: $U = 180$, $Z = 2.2$, $p = 0.026$) (Figure 4.5.31). Mercury content in seal vibrissae is strongly positively correlated with mercury content in fur ($R=0.75$, $p < 0.05$) (Figure 4.5.32). The correlation of mercury concentrations in vibrissae with age, weight, and body length is also positive, RS = 0.44, 0.49, and 0.52, respectively ($p < 0.05$) (Figure 4.5.33).

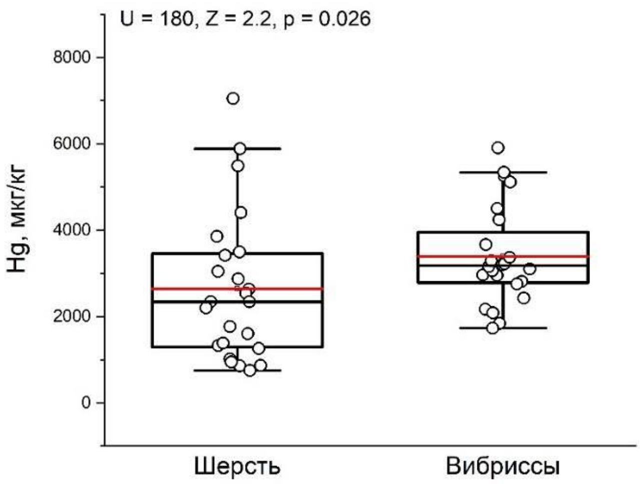


Figure 4.5.18 Comparative Mercury Content in Vibrissae of Male and Female Caspian Seals

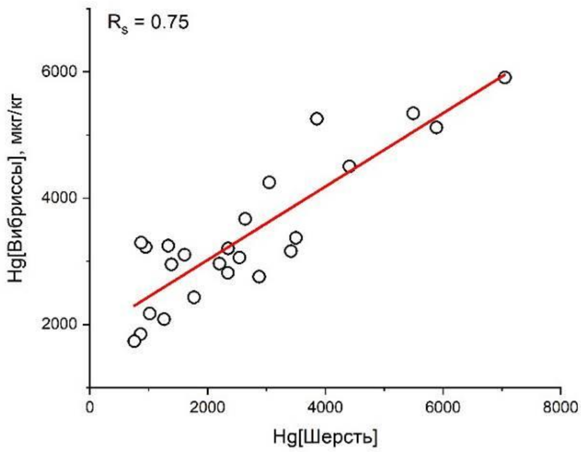


Figure 4.5.19 Relationship of Mercury Concentrations in Caspian Seal Vibrissae and Fur

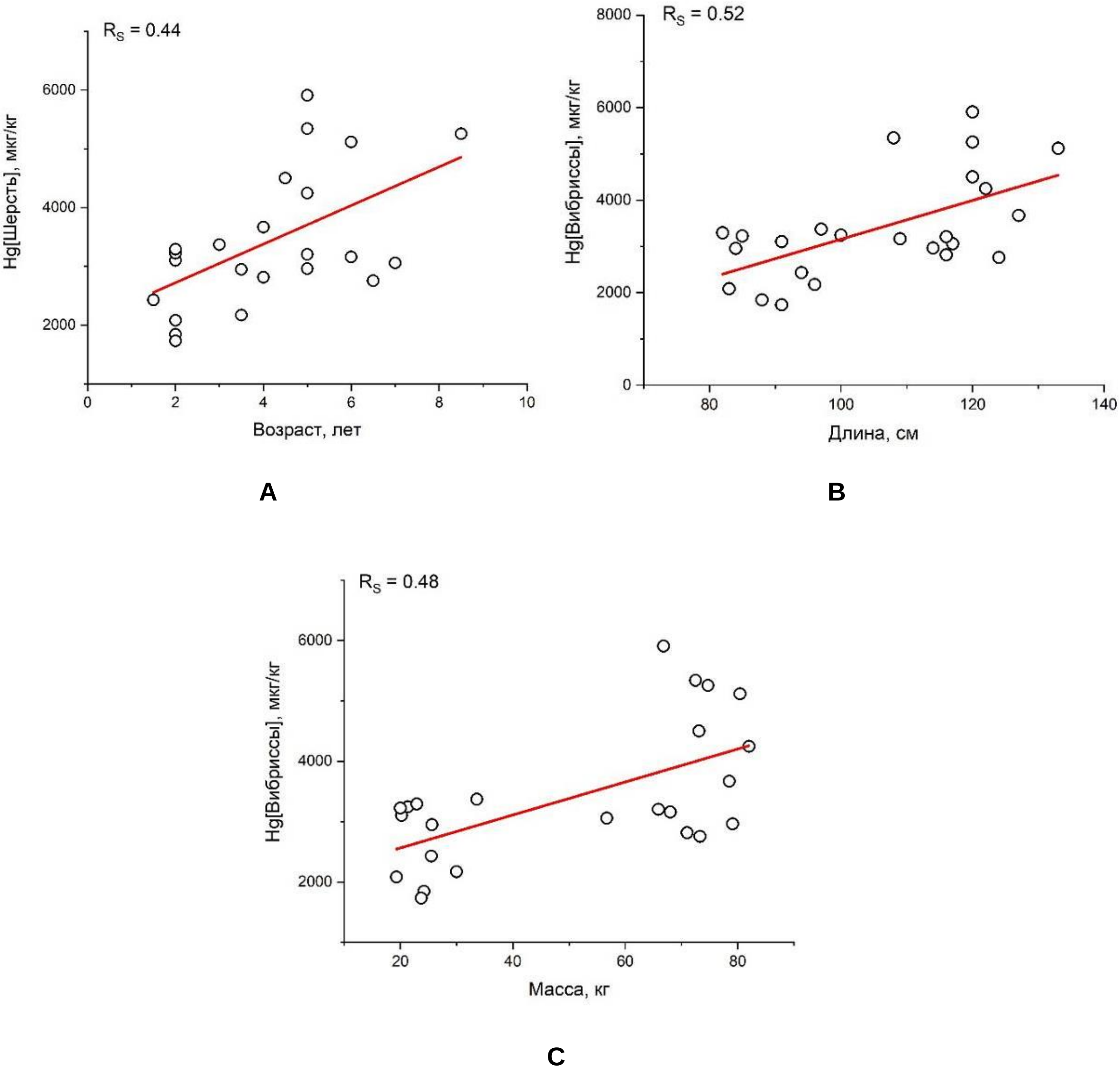


Figure 4.5.20 Mercury Content in Vibrissae of Caspian Seals Studied as a Function of Age (A), Length (B), and Body Weight (C)

4.5.3.3. Blood Mercury Content

The mean blood mercury concentration in Caspian seal blood was 106±34 µg/L and ranged from 54 to 184 µg/L. The frequency distribution of blood mercury concentrations is summarized in Table 4.5-7.

Table 4.5-4 Frequency Distribution of Mercury Concentrations in the Blood of the Caspian Seals Studied

Hg in blood, mcg/L	Frequency of occurrence, number of individuals	Cumulative frequency of occurrence, number of individuals	Percentage of total number	Cumulative percentage
0–50	0	0	0	0
50–100	9	9	39	39
100–150	12	21	52	91
150–200	2	23	9	100

There were no significant differences observed in blood mercury concentrations between males and females (M-W: $U = 24$, $Z = 1.1$, $p = 0.286$). Blood mercury concentration was positively correlated only with mercury in vibrissae ($R_S = 0.42$, $p < 0.05$) (Figure 4.5.34).

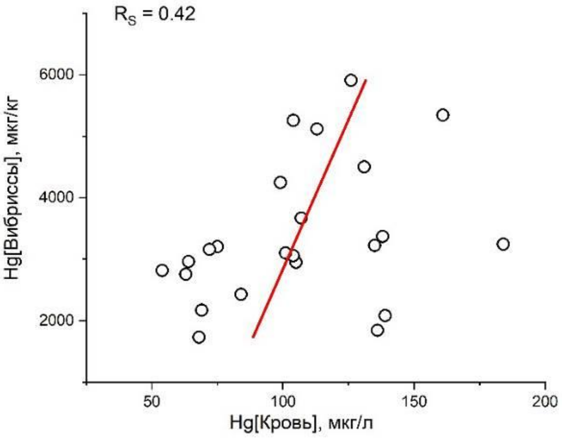
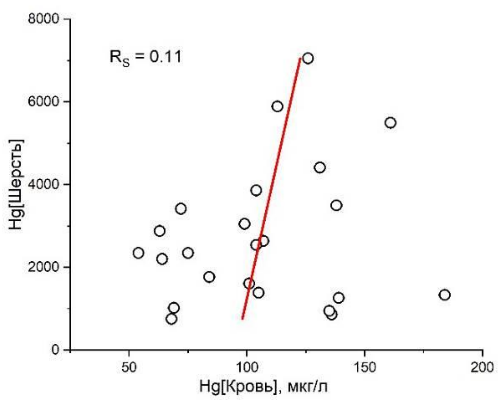
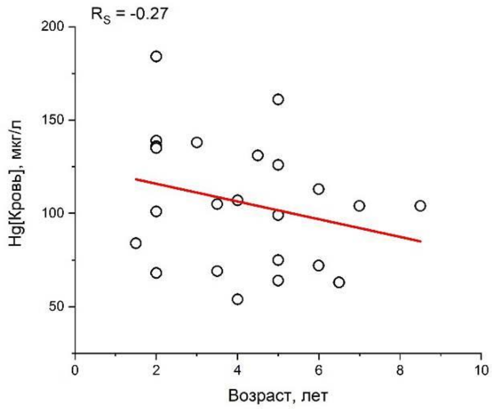


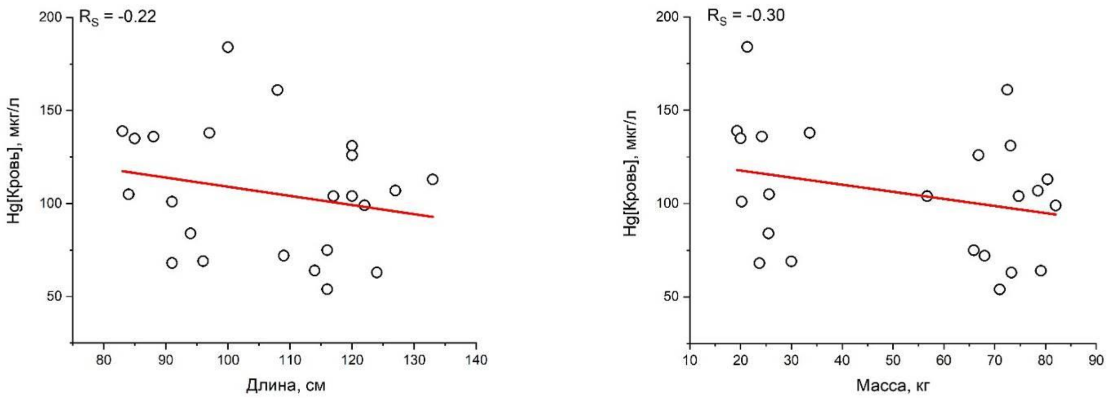
Figure 4.5.21 Correlation of Mercury Concentrations in Blood and Vibrissae of Caspian Seals

No statistically significant correlations were found between mercury concentrations in blood and mercury concentrations in hair, age, body length, and body weight of seals (Figure 4.5.35).





A B



C D

Figure 4.5.22 Blood Mercury Content of Caspian Harbor Seal as a Function of Mercury Content in Hair (A), Age (B), Body Length (C), and Body Weight (D) of the Animal

4.5.4. Hormonal Studies

4.5.4.1. Hormones in Blood

During the fieldwork, blood samples were collected from 20 Caspian seal individuals. The concentrations of three main steroid hormones (progesterone, testosterone, and cortisol) were analyzed in the blood serum of these animals. The results of the measurements are summarized in Table 4.5-8.

Table 4.5-5 Concentration of hormones in Caspian seal blood plasma

Animal No.	Sex	Age, years	Cortisol, ng/ml	Testosterone, ng/ml	Progesterone, ng/ml
PC23-01	female	4	326,25	0,24	153,50
PC23-02	female	4	1806,50	NA*	21,26
PC23-03	female	5	348,75	2,37	58,95
PC23-04	female	8	440,75	5,21	283,50
PC23-05	female	6	454,00	3,21	55,44
PC23-06	female	4	408,00	0,76	140,90
PC23-07	female	5	357,20	4,32	126,60
PC23-08	female	5	278,95	3,75	170,40
PC23-09	female	7	320,80	0,12	18,56
PC23-10	female	5	275,65	1,64	78,75
PC23-11	female	6	399,35	3,35	105,20
PC23-12	female	5	480,90	7,57	47,51
PC23-13	female	6	309,60	5,58	287,50
PC23-14	female	2	354,65	0,16	82,66
PC23-15	male	2	560,50	0,07	125,40
PC23-16	female	2	395,45	0,19	137,60
PC23-17	female	2	397,85	0,03	84,19
PC23-18	male	3	380,45	0,36	72,48
PC23-19	female	2	307,70	0,25	66,07
PC23-20	female	3	165,15	0,15	6,95
Mean value±se			**439,16±62,47**	**1,75±0,46**	**95,14±15,17**

**NA indicates a value below the sensitivity of the instrument and reagents*

Cortisol

Blood cortisol concentrations in Caspian seals ranged from 165.15 to 1806.5 ng/mL (mean±se: 439.16±62.47 ng/mL). No sex differences in blood cortisol concentration were found when comparing young males (n=2) and females (n=5) Caspian seals (W=9, p=0.41, Mann-Whitney test). Age differences between adult (n=13) and juvenile females (n=5) in blood cortisol concentration were not detected (W=50, p=0.76, Mann-Whitney test).

An individual with an extremely high blood cortisol level of 1806.5 ng/mL (PC23-02) was identified among individuals in the 2023 sample. Visually, the individual showed no health problems. Based on serological analysis, this individual has antibodies to 4 of the 8 diseases analyzed (see Section 4.5.5). However, other individuals with antibodies to 5 of the 8 diseases analyzed do not have such high cortisol levels. It is possible that these high cortisol levels are due to the individual response of this individual to the trapping procedures.

Testosterone

The concentration of testosterone in the blood of individuals captured in 2023 ranged from 0.03 to 7.57 ng/mL (mean±se: 1.75±0.46 ng/mL). No sex differences in blood testosterone concentration were found between young males (n=2) and females (n=5) (W=16, p=0.79, Mann-Whitney test). However, blood testosterone concentration in adult females (n=13) was higher than in young females (n=5) (W=75,p<0.01, Mann-Whitney test, (Figure 4.5.36).

Progesterone

Progesterone concentrations in the blood of individuals captured in 2023 ranged from 6.95 to 287.5 ng/mL (mean±se: 95.14±15.17 ng/mL). Sex differences in blood progesterone concentrations between young males (n=2) and females (n=5) were not detected (W=14, p=0.79, Mann-Whitney test), indirectly confirming the absence of sexual activity in young animals. Additionally, no differences were found in blood progesterone concentration between adult (n=13) and young females (n=5) (W=63, p=0.18, Mann-Whitney test).

Ultrasound and blood progesterone concentrations were compared to more accurately determine the presence of pregnancy. Females from the categories "adult pregnant females", "adult females probably not pregnant", "young females," and males were included in the analysis. There was no effect of pregnancy status on blood progesterone concentration (Kruskal-Wallis chi-squared=5.44, p=0.14, Kruskal-Wallis test, (Figure 4.5.37).

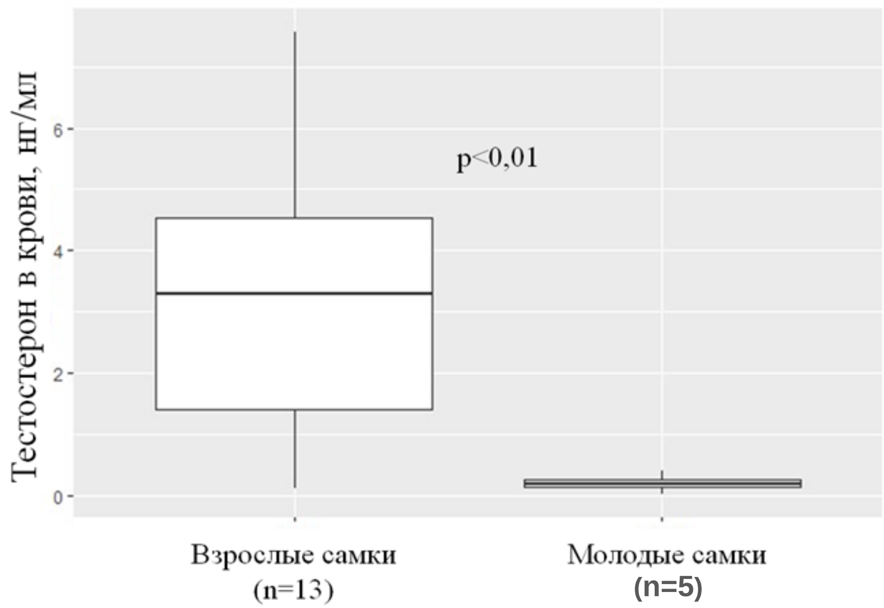


Figure 4.5.23 Blood testosterone concentrations in adult and juvenile female Caspian seals

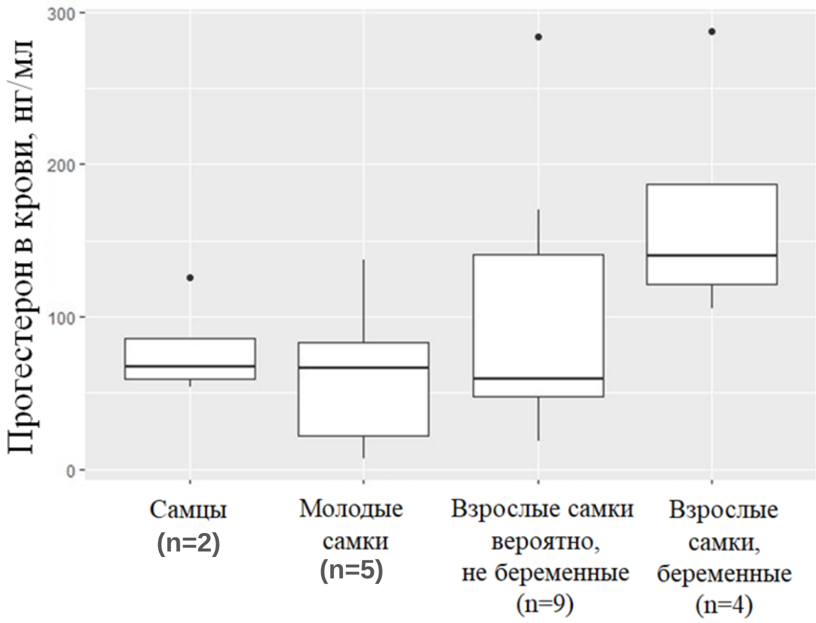


Figure 4.5.24 Blood Progesterone Concentrations in Caspian Seals with Different Pregnancy Statuses

4.5.4.2. Hormones in Hair

Hair samples were collected from 20 Caspian seals (hair from the waist and withers). The concentrations of three steroid hormones (cortisol, testosterone, and progesterone) in Caspian seal hair are summarized in Table 4.5-9.

Table 4.5-6 Concentrations of Hormones in Caspian Seal Hairs

Animal Number	Hair from the Loin Area			Hair from the Withers Area		
	Cortisol, ng/g	Testosterone, ng/g	Progesterone, ng/g	Cortisol, ng/g	Testosterone, ng/g	Progesterone, ng/g
PC23-01	4,28	0,38	3,79	6,35	0,56	2,78
PC23-02	11,84	1,09	9,49	8,02	1,35	9,66
PC23-03	16,84	0,93	8,88	11,25	1,36	10,09
PC23-04	30,64	0,85	14,83	46,97	1,55	19,76
PC23-05	14,08	0,93	11,53	36,64	1,43	18,72
PC23-06	69,87	0,84	10,14	31,27	1,45	15,29
PC23-07	21,61	0,75	23,17	3,93	0,55	5,75
PC23-08	11,60	0,60	15,61	NA*	0,69	4,09
PC23-09	4,51	0,71	9,14	9,82	0,68	6,65
PC23-10	47,37	1,03	13,02	43,75	1,69	53,01
PC23-11	9,51	0,67	9,57	10,00	1,31	12,40
PC23-12	139,40	1,42	20,16	11,76	1,02	9,53
PC23-13	17,96	0,83	12,15	9,40	0,99	9,65
PC23-14	22,20	0,41	6,97	NA	0,48	3,66
PC23-15	NA	3,35	15,99	16,12	1,09	9,02
PC23-16	223,61	1,26	NA	9,35	0,37	1,66
PC23-17	53,59	1,04	6,48	743,97	3,05	30,41
PC23-18	37,55	1,14	8,72	7,62	0,89	7,05
PC23-19	464,60	2,83	19,22	172,14	1,29	29,84
PC23-20	38,40	1,07	19,66	26,30	0,43	18,77
Mean value±se	**98,24±39,93**	**1,23±0,17**	**13,00±1,29**	**67,27±36,70**	**1,19±0,16**	**14,91±2,78**

**The value is below the sensitivity level of the instrument.*

Hormones in Hair from Different Body Sites

The Wilcoxon test for matched pairs (wilcox.test) was used to compare the concentrations of hormones in hair collected from different parts of the Caspian seal body (withers and loins). No statistically significant differences were found in the concentrations of all three hormones (cortisol, testosterone, and progesterone) in hair from the withers and loin area (p>0.05) (Figure 4.5.38). In this paper, the concentrations of hormones in hair collected from the lumbar region will be used for further comparisons.

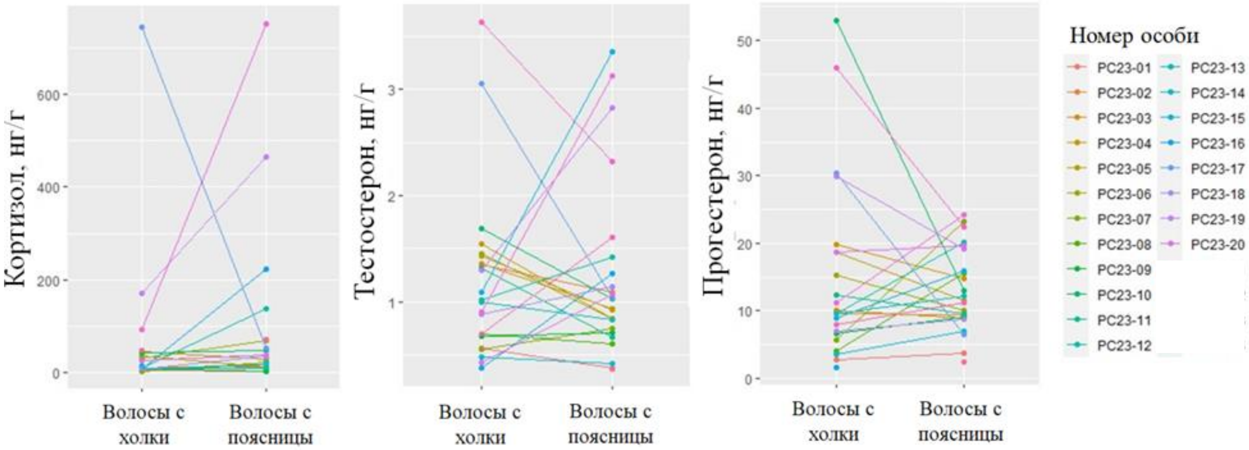


Figure 4.5.25 Hormone Concentrations in Caspian Seal Hair Collected from the Withers and Loin Region

Cortisol

Cortisol concentrations in hair collected from the loin region ranged from 4.28 to 752.25 ng/g (mean±se: 98.24±39.93 ng/g), while in hair collected from the withers ranged from 3.93 to 743.97 ng/g (mean±se: 67.27±36.70 ng/g). No sex differences in hair cortisol concentration were found when comparing young males (n=2) and females (n=5) of Caspian seals (W=6, p=0.5, Mann-Whitney test). Young females (n=5) have higher hair cortisol concentrations than adult females (n=13) (W=10, p=0.0034, Mann-Whitney test) (Figure 4.5.39).

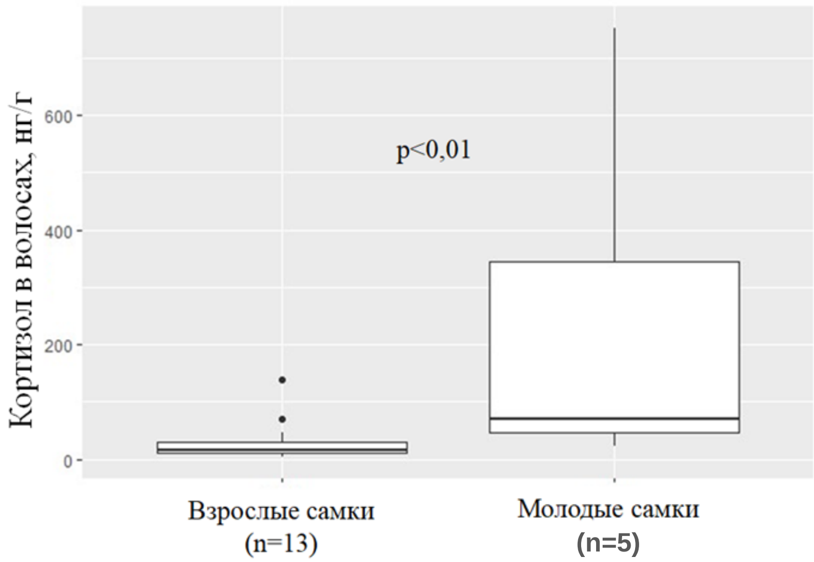


Figure 4.5.26 Cortisol concentration in the hair of adult and juvenile female Caspian seals

Testosterone

Hair testosterone concentrations collected from the lumbar region ranged from 0.37 to 3.35 ng/g (mean±se: 1.23±0.17 ng/g), while those collected from the withers ranged from 0.37 to 3.64 ng/g (mean±se: 1.19±0.16 ng/g).

No sex differences in hair testosterone concentrations were found between young males (n=2) and females (n=5) (W=7, p=0.23, Mann-Whitney test). Likewise, no age differences were found in hair testosterone concentrations between adult females (n=13) and young females (n=5) (W=30, p=0.24, Mann-Whitney test).

Progesterone

Hair progesterone concentrations collected from the lumbar region ranged from 2.37 to 24.23 ng/g (mean±se: 13.00±1.29 ng/g), whereas those collected from the withers ranged from 1.66 to 53.01 ng/g (mean±se: 14.91±2.78 ng/g).

No sex differences were found in hair progesterone concentrations between young males (n=2) and females (n=5) (W=10, p=0.76, Mann-Whitney test). Additionally, no age differences were found in hair progesterone concentrations between adult females (n=13) and young females (n=5) (W=41, p=0.90, Mann-Whitney test).

4.5.4.3. Hormones in Vibrissae

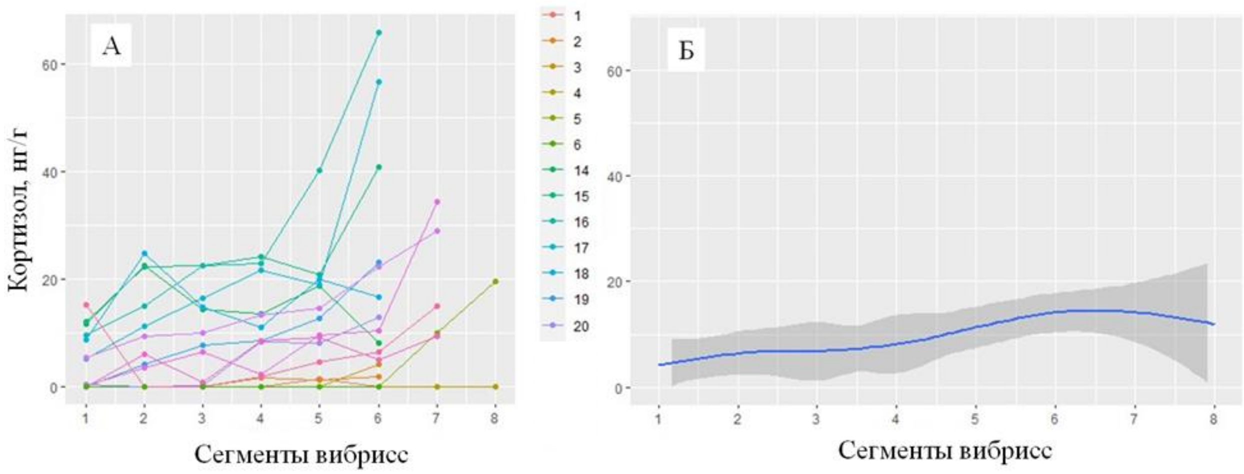
Concentrations of three hormones (cortisol, progesterone, and testosterone) were measured in vibrissae segments of 13 Caspian seal specimens (PC23-01-06; PC23-14-20) (Table 4.5-10). Cortisol concentrations ranged from 0.06 to 66.01 ng/g (mean±se: 13.88±1.42 ng/g), testosterone from 0.12 to 1.09 ng/g (mean±se: 0.42±0.02 ng/g), and progesterone from 0.46 to 24.42 ng/g (mean±se: 5.39±0.39 ng/g).

Table 4.5-7 Concentration of hormones in segments of Caspian seal vibrissae

Animal No.	Sex	Age	Vibrissae segment	Cortisol, ng/ml	Testosterone, ng/ml	Progesterone, ng/ml
PC23-01	female	4	1	0	0,26	3,27
			2	0	0,59	7,23
			3	0	0,59	7,74
			4	0	0,48	6,64
			5	1,51	0,61	6,37
			6	0	0,56	5,70
			7	0	0,65	7,52
PC23-02	female	4	1	0	0,48	3,15
			2	0	0,19	2,79
			3	0	0,53	4,18
			4	1,70	0,83	7,26
			5	1,15	0,87	9,33
			6	1,94	0,61	5,84
PC23-03	female	5	1	0	0,31	3,45
			2	0	0,48	3,98
			3	0	0,59	2,89
			4	0	0,37	2,97
			5	0	0,44	3,42
			6	4,15	0,63	3,63
PC23-04	female	8	1	0	0,30	3,09
			2	0	0,33	4,07
			3	0	0,39	4,90
			4	0	0,65	6,86
			5	0	0,71	6,12
			6	0	0,54	6,44
			7	0	0,75	9,21
			8	0	0,87	7,55
PC23-05	female	6	1	0	0,20	3,51
			2	0	0,31	3,11
			3	0	0,39	2,72
			4	0	0,58	3,55
			5	0	0,86	5,03

Animal No.	Sex	Age	Vibrissae segment	Cortisol, ng/ml	Testosterone, ng/ml	Progesterone, ng/ml
			6	0	0,39	2,59
			7	10,00	0,60	3,12
			8	19,55	0,56	3,91
PC23-06	female	4	1	0	0,16	1,95
			2	0	0,19	2,26
			3	0	0,17	1,72
			4	0	0,20	1,97
			5	0	0,23	2,35
			6	0	0,28	2,66
PC23-14	female	2	1	11,67	0,40	4,22
			2	22,60	0,55	7,05
			3	14,31	0,47	6,35
			4	13,55	0,73	10,72
			5	18,67	1,08	14,78
			6	8,15	0,39	4,56
PC23-15	male	2	1	12,12	0,27	0,77
			2	22,30	0,43	2,46
			3	22,50	0,47	3,05
			4	24,14	0,46	7,74
			5	20,75	0,49	6,96
			6	40,80	0,51	6,12
PC23-16	female	2	1	9,60	0,30	1,70
			2	14,97	0,39	2,77
			3	22,54	0,51	3,56
			4	22,95	0,61	6,74
			5	40,28	1,09	24,42
			6	66,01	0,68	10,85
PC23-17	female	2	1	5,27	0,17	4,09
			2	11,25	0,30	3,84
			3	16,41	0,44	2,37
			4	21,63	0,71	7,75
			5	18,98	0,76	18,44
			6	56,76	0,70	19,03
PC23-18	male	3	1	8,82	0,26	1,96
			2	24,89	0,25	2,66
			3	14,68	0,47	8,39
			4	10,92	0,51	7,45
			5	19,96	NA	17,45
			6	16,60	0,56	5,04
PC23-19	female	2	1	0	0,28	1,27
			2	4,17	0,24	2,36
			3	7,68	0,24	3,30
			4	8,63	0,34	10,29
			5	12,74	0,52	16,51
			6	23,17	0,38	10,32
PC23-20	female	3	1	0,48	0,14	0,67
			2	0	0,15	0,46
			3	0,09	0,12	0,55
			4	8,42	0,16	1,24
			5	8,20	0,24	6,16
			6	12,81	0,28	3,57
Mean value±se				**9,13±1,12**	**0,42±0,02**	**5,39±0,39**

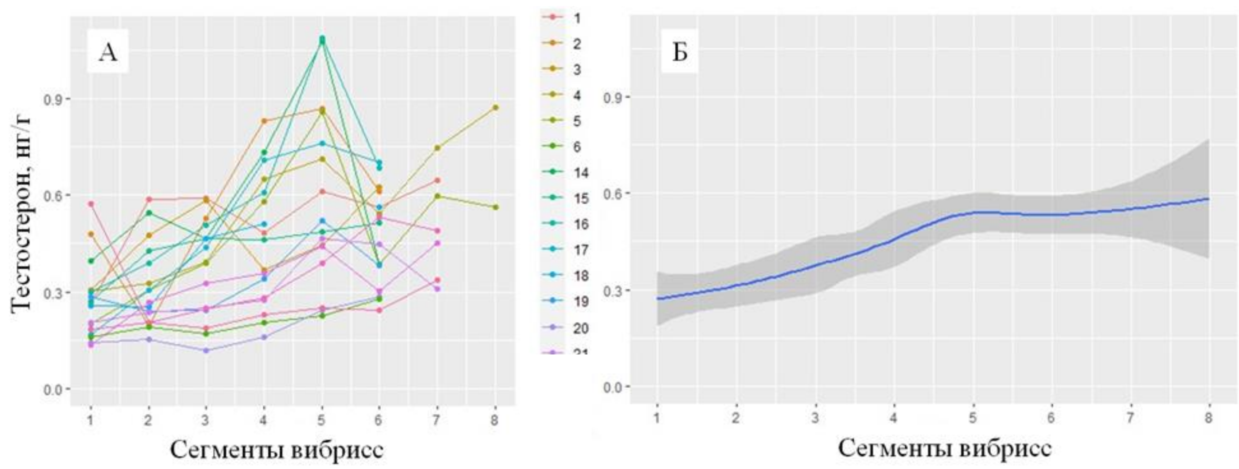
The distribution pattern of cortisol and testosterone along the vibrissae shows an increase in the concentrations of these hormones closer to the distal end of the vibrissae (Figures 4.5.40-4.5.41).



A - distribution of cortisol along vibrissae in each specimen of the sample,

B - smoothed mean and confidence interval of cortisol distribution (function gglot, geom_smooth)

Figure 4.5.27 Cortisol concentration in segments of the Caspian seal vibrissae

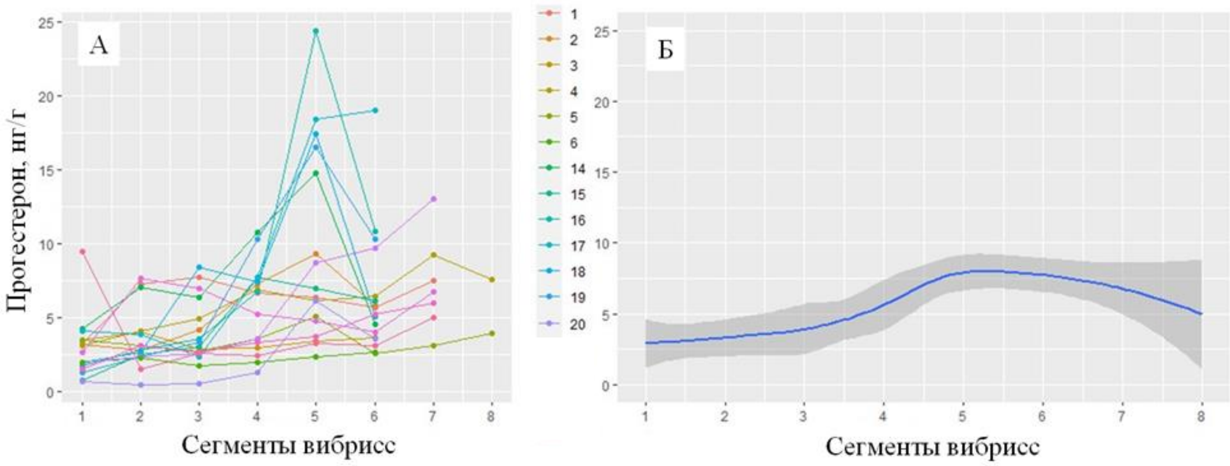


A - distribution of testosterone along the vibrissae of each sampled individual,

B - smoothed mean and confidence interval of testosterone distribution (using the ggplot function, geom_smooth)

Figure 4.5.28 Testosterone concentration in segments of the Caspian seal vibrissae

In the distribution of progesterone along the vibrissae, a peak is noticeable in the 5th vibrissae segment (Figure 4.5.42).



A - distribution of progesterone along the vibrissae of each sampled individual,

B - smoothed mean and confidence interval of progesterone distribution (using the ggplot function, geom_smooth)

Figure 4.5.29 Progesterone concentration in segments of the Caspian seal vibrissae

Sex and age characteristics

Based on ultrasound results and blood progesterone concentrations, we categorized pregnant females (adult females with blood progesterone concentrations above 100 ng/mL) and separated the 2023 sample by sex and age characteristics. The 2023 sample contained 2 young males, 5 young females, 6 adult female seals, and 7 adult pregnant females. Adult males were not present in the 2023 sample.

Age differences were evident in the pattern of cortisol distribution along the vibrissae - young males and females had higher cortisol concentrations in the vibrissae than adult female seals and pregnant females (Figure 4.5.43). Cortisol concentrations in the vibrissae of pregnant females were low, close to 0 ng/mL; in adult female seals, low cortisol values were characteristic of vibrissae segments 1-5, with concentrations increasing in more distal segments.

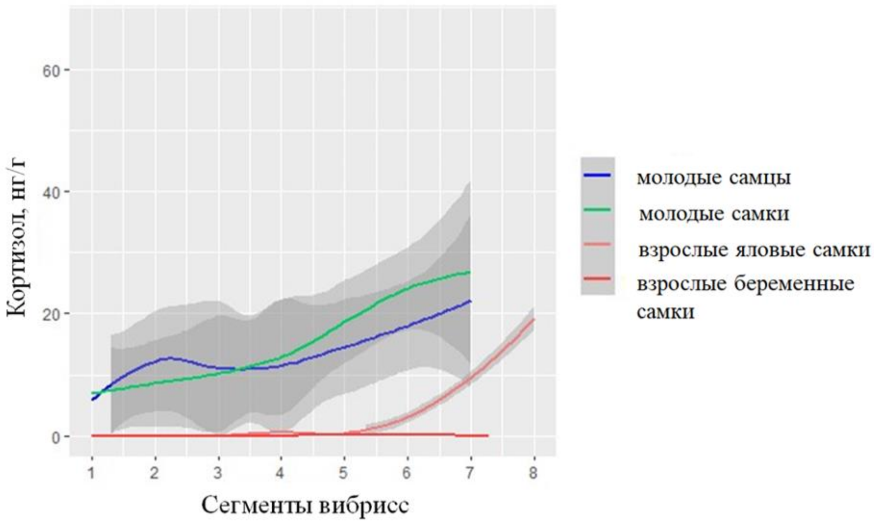


Figure 4.5.30 Cortisol concentrations in Caspian seal vibrissae segments of different sexes, ages, and pregnancy statuses

Young males and adult females showed an increase in testosterone concentration closer to the distal end of the vibrissae, with a particularly pronounced increase in concentration characteristic of pregnant females (Figure 4.5.44). Young females showed a peak of testosterone at the 5th-6th segment of vibrissae.

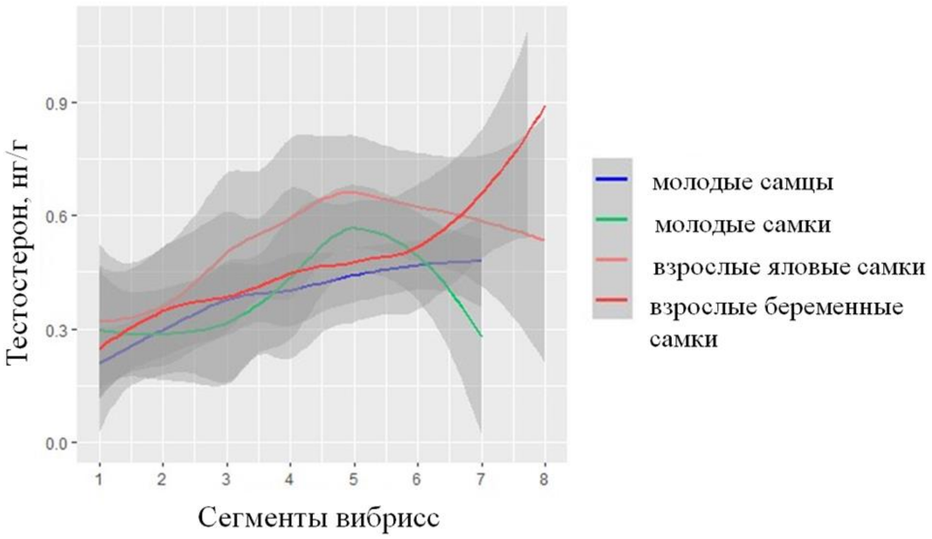


Figure 4.5.44 Concentration of testosterone in segments of the vibrissae of Caspian seals of different sexes, ages, and pregnancy statuses.

The distribution of progesterone in the vibrissae of young females showed a peak in segments 5-6 (Figure 4.5.45). In the vibrissae of young males and adult females, the concentration of progesterone is more evenly distributed.

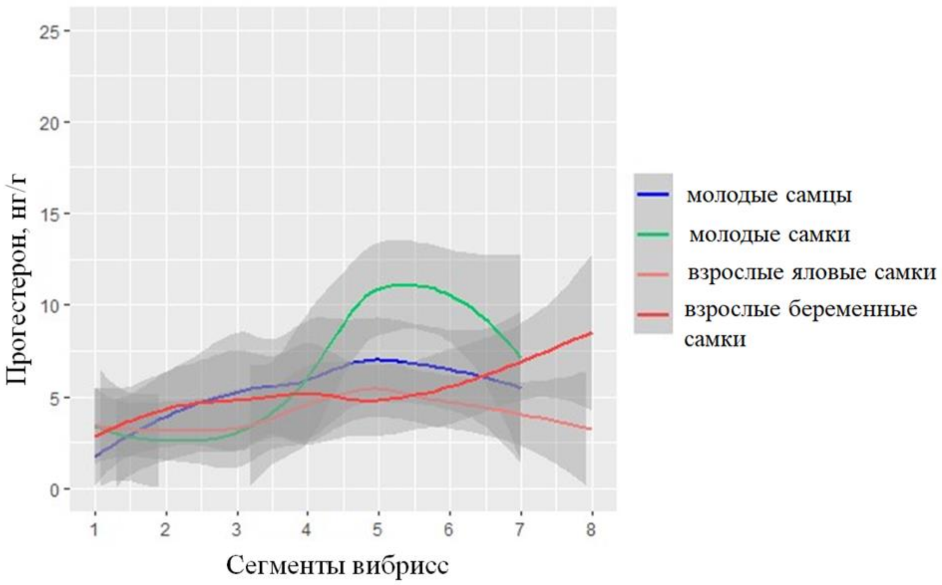


Figure 4.5.45 Concentration of testosterone in segments of the vibrissae of Caspian seals of different sexes, ages, and pregnancy statuses

4.5.5. Serological studies

Serological studies were performed on 20 serum samples from seals of different ages. All samples were analyzed for the presence of antibodies to herpes simplex virus, carnivore plague virus, Toxoplasma gondii, mycoplasma, chlamydia, candida, and Trichinella sp. (Table 4.5-11).

Table 4.5-8 Seropositivity of Caspian seals to different pathogens

Specimen No.	Herpes simplex virus	Toxoplasma gondii	Chlamydia sp.	Mycoplasma sp.	Candida sp.	Morbillivirus	Trichinella sp.	Number of positive results
PC23-01	negative	positive	positive	positive	positive	positive	negative	5
PC23-02	negative	negative	positive	positive	positive	positive	negative	4
PC23-03	negative	negative	negative	negative	negative	positive	negative	1
PC23-04	negative	negative	dubious	dubious	positive	positive	negative	2
PC23-05	negative	positive	positive	positive	positive	positive	negative	5
PC23-06	negative	negative	positive	positive	positive	positive	negative	4
PC23-07	negative	negative	positive	positive	positive	positive	negative	4
PC23-08	negative	positive	positive	positive	positive	positive	negative	5
PC23-09	negative	negative	positive	positive	positive	positive	negative	4
PC23-10	negative	negative	dubious	dubious	dubious	dubious	negative	0
PC23-11	negative	negative	negative	positive	positive	dubious	negative	2
PC23-12	negative	dubious	positive	positive	positive	positive	negative	4
PC23-13	negative	negative	positive	positive	positive	positive	negative	4
PC23-14	negative	negative	positive	positive	positive	positive	negative	4
PC23-15	negative	negative	positive	positive	positive	positive	negative	4
PC23-16	negative	negative	positive	positive	positive	dubious	negative	3
PC23-17	negative	negative	positive	negative	positive	negative	negative	2
PC23-18	negative	dubious	positive	positive	positive	positive	negative	4
PC23-19	negative	negative	positive	positive	positive	positive	negative	4
PC23-20	negative	negative	positive	negative	positive	dubious	negative	3

No animals were seropositive for herpes simplex virus and Trichinella in 2023. Seropositive animals were identified for the remaining five pathogens. Seropositivity to Toxoplasma was minimal (13%), while seropositivity to the other pathogens was significantly higher: 67% for HPV, 71% for mycoplasma, 79% for chlamydia, and 92% for candida (Table 4.5-12).

Table 4.5-9 Summary of seropositivity to different pathogens

Pathogene	Number of tests	Number of positive results	Seropositive, %
Toxoplasma gondii	24	3	13
Herpes simplex virus	24	0	0
Trichinella sp.	24	0	0
Morbillivirus	24	16	67
Mycoplasma sp.	24	17	71
Chlamydia sp.	24	19	79
Candida sp.	24	22	92

Only one seal, PC23-10, was not positive for all seven pathogens. Two seals were seropositive for one pathogen, four seals were seropositive for two pathogens, three seals were seropositive for three pathogens, and the most animals seropositive for four pathogens simultaneously were 10 animals. Three animals were seropositive for five pathogens simultaneously."

4.5.6. Immunity studies

When analyzing the parameters of the immune system, no significant differences were found in the activity of the complement system (with an average titer of 3-3.4 in the bacterial killing test for animals of different age groups), immunoglobulin G levels (ranging from 53.8 to 60.1 mg/ml), and total immunoglobulin activity in the hemagglutination test with ram's erythrocytes (consistently measuring 0 in all animals). However, significant differences were noted only in the concentration of lysozyme, which was significantly lower in the two other age groups of animals (see Figure 4.5.46).

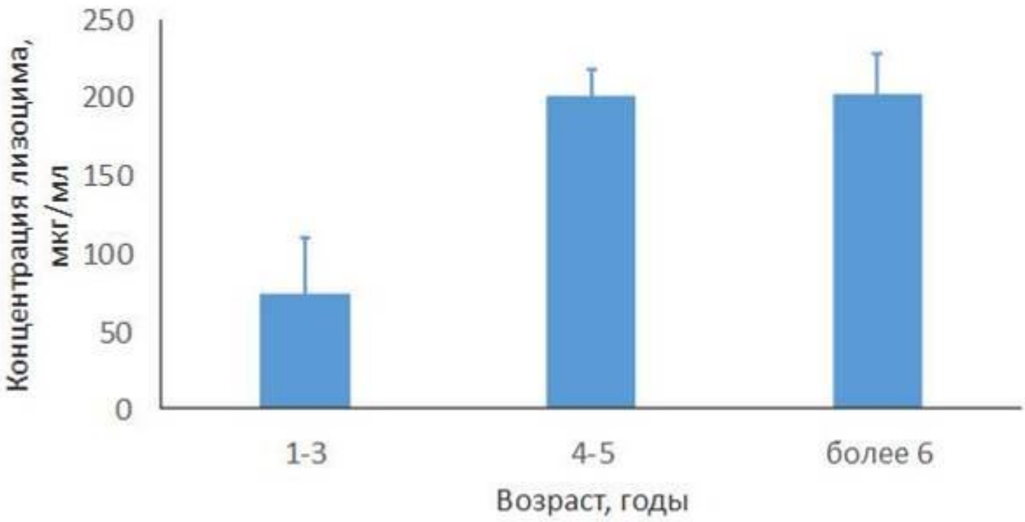


Figure 4.5.31 Lysozyme levels in Caspian seals of different age groups

5. COMPARATIVE ANALYSIS OF THE OBTAINED RESULTS WITH THE DATA FROM LITERATURE SOURCES

5.1. Population Accounting

During the voyage of the R/V "Nautilus-one" (November 7, 2023, to November 16, 2023) in the Kazakhstan water area of the Caspian Sea, 200 sightings of Caspian seals (totaling 217 individuals) were recorded."

In preparation for seal capture, six sand islands were inspected, out of which four (KT1 to KT4) were submerged due to surges and rising sea levels (refer to Table 4.2-1). Caspian seal haul-outs were observed at KT5 and KT6, where 9836 Caspian seals were counted over two days of trapping. Additionally, four dead seals were recorded en route to the KT2 sand island.

Table 5.1-1 summarizes the calculation of the density of Caspian seals encountered in the Kazakhstan part of the Northern Caspian Sea during the 2019-2020 and 2022-2023 surveys.

Table 5.1-1 Results of the Caspian seal population count in the water area

Date	Obseravtion point	Route length*, km	Width of Survey Transect (km)	Area of Survey Transect (km²)	Number of counted seals, individuals	Density, individuals/ km2
2019						
04.11.19	SRV Alina	67	3	201,0	24	0,12
09.11.19	SRV Alina	83	3	261,0	25	0,10
Average number as recorded from vessel						**0,11**
04.11.19	Motor boat	26	0,7	18,2	76	4,18
07.11.19	Motor boat	31	0,7	21,7	189	8,71
08.11.19	Motor boat	34	0,6	20,4	216	10,59
Average number as recorded from boat						**7,82**
2020						
01.11.20-03.11.20	SRV Alina	282	1	282	44	0,15
03.11.20	Motor boat	26	0,6	15,6	88	5,64
06.11.20	Motor boat	26	0,6	15,6	142	9,10
Average number as recorded from boat						**7,37**
2022						
03.11.22	MV Nautilus-one	96	1	96,0	29	0,30
04.11.22	Motor boat	15,6	0,8	12,5	41	3,28
	Motor boat	15,6	0,8	12,5	90	7,20
05.11.22	Motor boat	15,6	0,8	12,5	56	4,48
06.11.22	Motor boat	15,6	0,8	12,5	21	1,68
07.11.22	Motor boat	15,6	0,8	12,5	42	3,36
08.11.22	Motor boat	15,6	0,8	12,5	34	2,72
Average number as recorded from boat						**3,79**
2023						
11.11.2023	MV Nautilus-one	217	1	217	147	0,68
16.11.2023	MV Nautilus-one	118	1	118	70	0,59
11.11.2023	Motor boat	34	0,8	27,2	18	0,66
13.11.2023	Motor boat	24,6	0,8	19,7	46	4,5
14.11.2023	Motor boat	8,0	0,8	6,4	60	9,4
Average number as recorded from boat						**4,85**

Note: ** indicates the total length of the route segments where observations were conducted."*

Surveys in shallow water areas (offshore sand islands north of Kulaly Island and Morsky Island, north and south of the NCMC, at depths of 0.5-4 m) show similar results to the data from 2019-2022. Surveys in shallow water areas (at sea sand island to the north of Kulaly and Morskoye islands, to the north and south of SCMC, at depths of 0.5-4 m) conducted from motorboats in 2023 exhibit similar results to the data from 2019-2022, ranging from 0.68 to 9.4 eq/km2. Surveys from the research vessel conducted between 2019 and 2023 demonstrate a gradual increase in Caspian seal density along the vessel's route from Bautino to the Sedlovina section and further into the North Caspian Marine Channel (NCMC) area. Seal density increased from 0.12 eq/km2 to 0.68 eq/km2, likely due to the shallowing of coastal areas and the gradual change in fish migration routes towards deep water, west of Kulaly Island (comprising common sprat and mullet).

5.2. Seal Tracking

Satellite transmitters were deployed on Caspian seals during the study period: 9 transmitters in 2019, 11 transmitters in 2020, 20 tags (on female Caspian seals) in 2022, and 13 tags (on female Caspian seals) in 2023. The tagging area in 2019, 2020, and 2023 was located approximately 200-250 km northeast of the 2022 tagging area.

Analysis of the obtained data revealed some similarities and differences in the indicators. The ability of animals to move long distances southward was noted for all years of observation, with individual animals in 2020, 2022, and 2023 moving into the southern Caspian Sea during the tracking period (refer to Figure 5.2.1). Seals were observed to actively use the waters of the middle Caspian Sea throughout all years and months of the survey.

In 2019 and 2022, the animals practically did not use the western part of the Caspian Sea for movements, while in 2020, individuals were recorded in the area of Tyuleniy Island and along the western coast of the Caspian Sea in the Republic of Dagestan."In 2022, a significant number of locations were confined to the eastern coast of the Caspian Sea, while in 2020 only 2 individuals actively used this part of the water area, and in 2019 seals were practically not recorded along the eastern coast of the Caspian Sea.

"In 2023, the animals utilized both the western and eastern shores of the water area for movements. In 2022 and 2023, water areas adjacent to the east coast were predominantly utilized.

Comparison of the years 2019, 2020, 2022, and 2023 by month reveals the following:

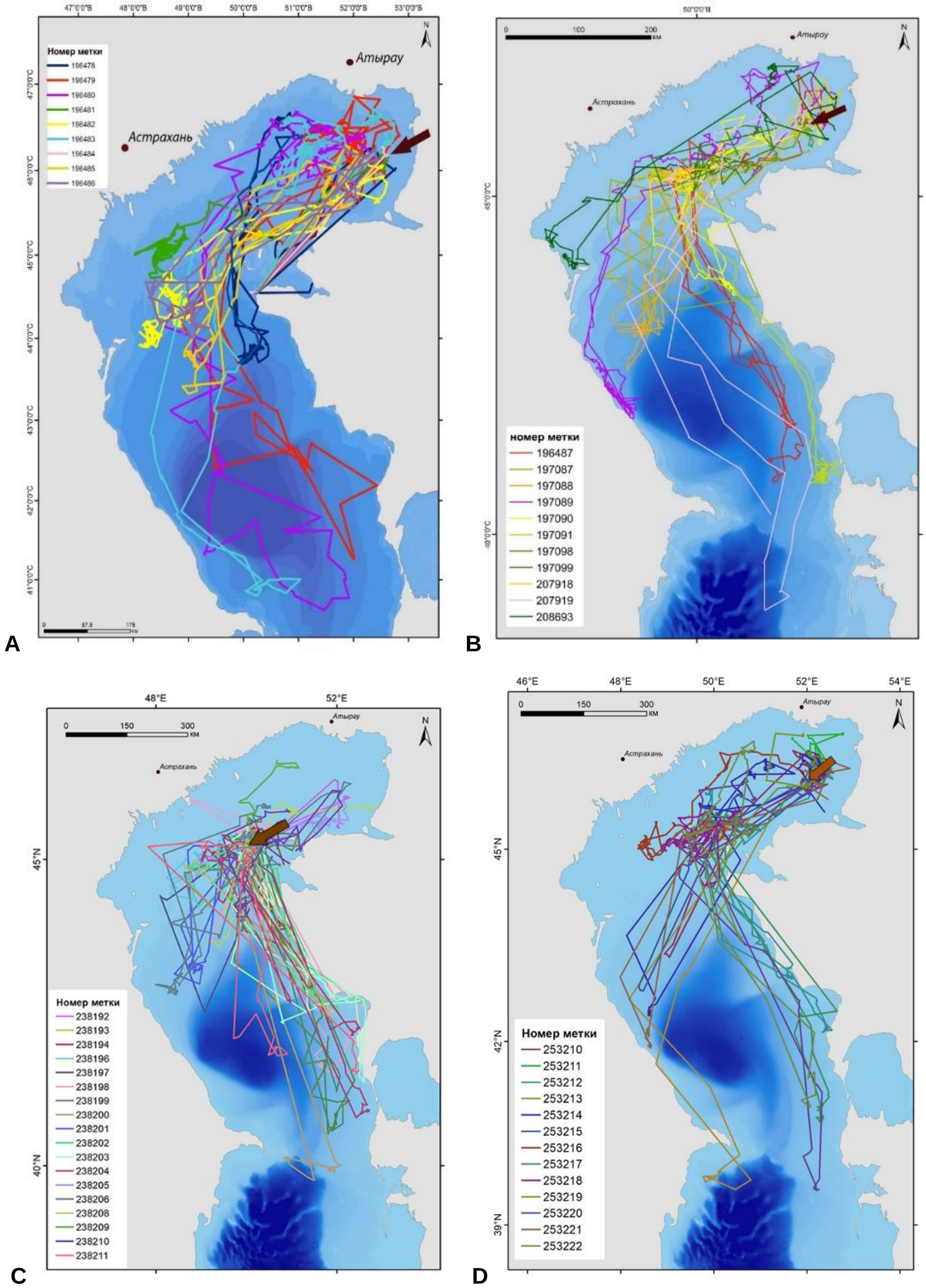
- In November, the primary key habitat area was situated in the tagging area. In 2019 and 2020, individual animals migrated into the southwestern part of the northern Caspian Sea and the middle Caspian Sea in November. In 2022 and 2023, animals also ventured into the middle Caspian Sea, utilizing, among other areas, the water area near the eastern coast (refer to Figure 5.2.2, A, B, C, D).
- In December (Figure 5.2.2, H), January (Figure 5.2.2, L), and February (Figure 5.3.2, P), the location of key sites in 2023 resembled that of the 2020 and 2022 tagging, situated in the Seal Island area for both years.

Additionally, for all months during the 2020 and 2022 tagging periods, there were movements of animals into the middle Caspian Sea. It is also noted that in December and January of those years, seals migrated along the eastern coast."

"It should be noted that in 2022, animals practically did not utilize the western part of the Caspian Sea water area, in contrast to 2019 and 2020. The movements of animals tagged in 2019 differ significantly - for that year's tagging, there is a shift of large key habitat areas to the northeastern part of the Caspian Sea. Movements in March between the three tagging years cannot be compared due to the small number of locations obtained in March for the 2019 and 2020 tags.

For all the indicated months during the tagging years 2020, 2022, and 2023, animal movements into the middle Caspian Sea are noted. In December and January of the mentioned years, seals moved along the eastern coast. Additionally, in January 2024, animals also utilized the western part of the water area for movements.

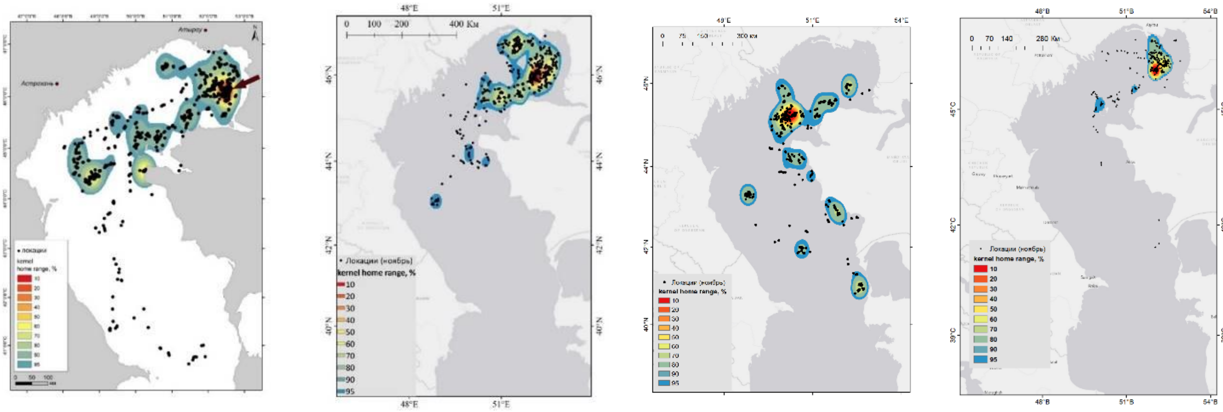
In contrast to animals tagged in 2019, 2020, and 2023, which utilized the western part of the Caspian Sea area, seals tagged in 2022 practically did not use this part for movements. The movements of animals tagged in 2019 are notably different - for that year's tagging, there is a shift of large key habitat areas to the northeastern part of the Caspian Sea."



A - in 2019, B - in 2020; C - in 2022, D - in 2023

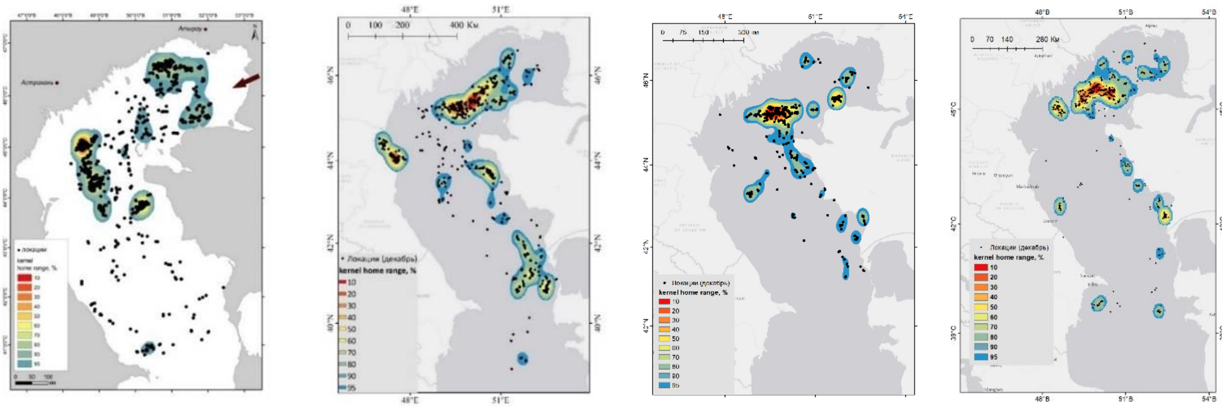
Figure 5.2.1 Tracks of tagged seals

November



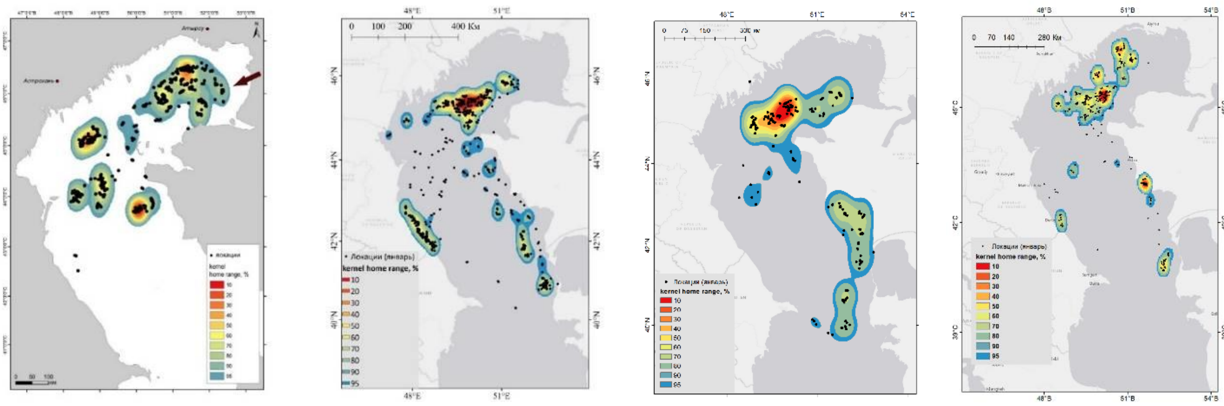
A *B* *C* *D*

December



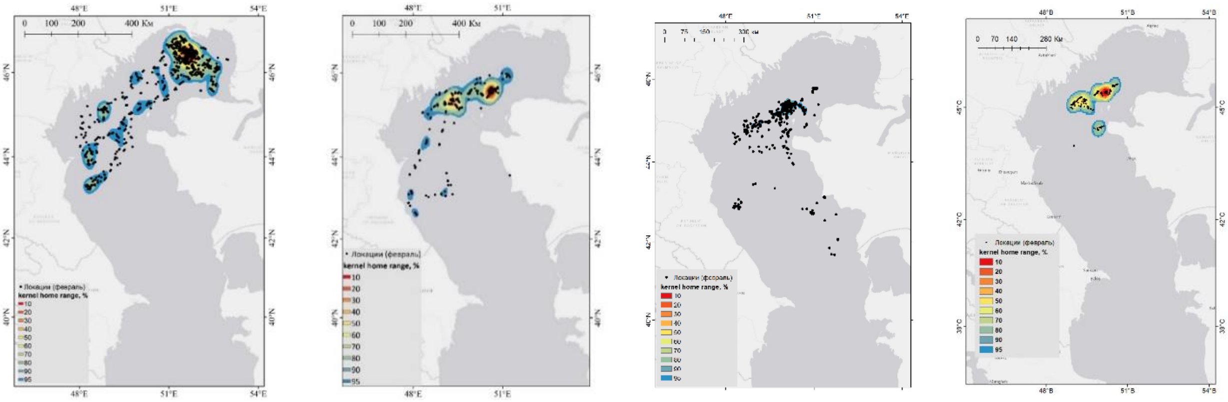
E *F* *G* *H*

January



I *J* *K* *L*

February



M ***N*** ***O*** ***P***

A - in November 2019; B - in November 2020; C - in November 2022; D - in November 2022;

E - in December 2019; F - in December 2020; G - in December 2022; H - in December 2023;

I - in January 2020; J - in January 2021; K - in January 2023; L - in January 2024;

M - in February 2020; N - in February 2021; O - in February 2023; P - in February 2024.

Figure 5.2.2 Key habitat areas for tagged seals

Probably, the observed peculiarities of the location of key areas are related, as before, to the peculiarities of ice conditions and the location of ice fields - the distribution of Caspian seals in the winter period strongly depends on ice conditions [Ivanov and Sokolsky, 2000].

"The winter of 2019-2020 was characterized by an extremely low degree of ice cover development, with ice barely covering the northernmost part of the Caspian Sea (refer to Figure 5.2.3 A). Apparently, in 2019, the distribution of seals was closely related to the location of ice fields, which were near the northern coast that year.

Meteorological conditions during the winter seasons of 2020-2021, 2022-2023, and 2023-2024 were more typical, with ice covering most of the North Caspian Sea.

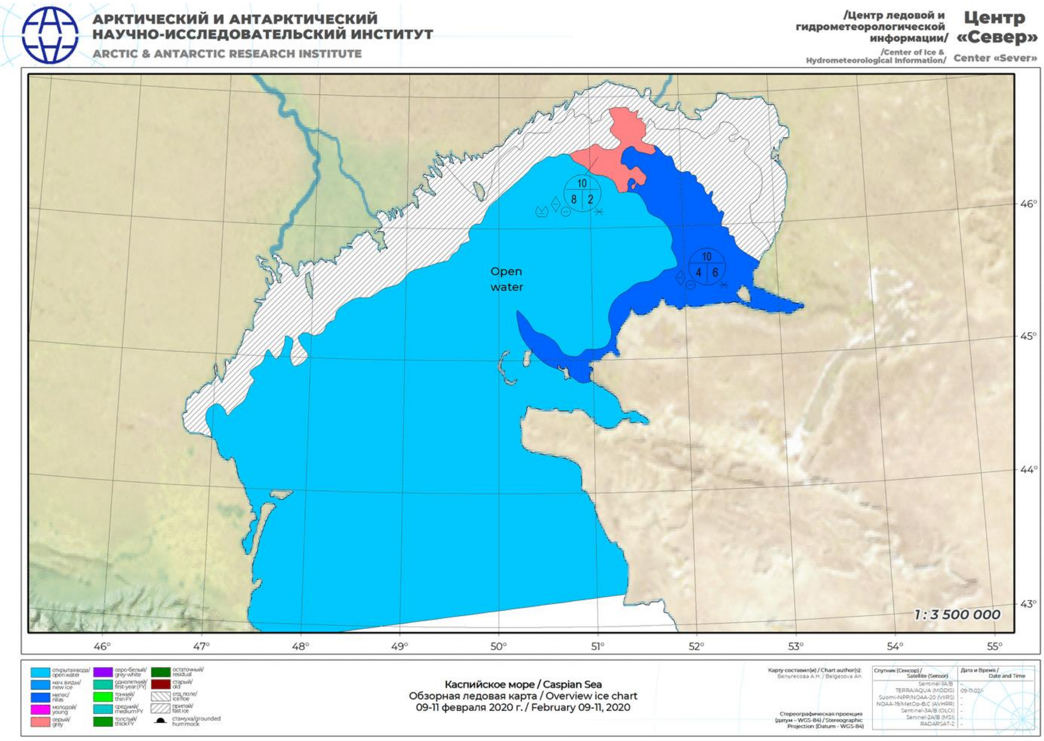
In years with normal sea ice extent, the Mangyshlak Sill appears to be ice-covered, providing suitable conditions for wintering and breeding seals (refer to Figure 5.2.3 B, C). Additionally, hydrological and geomorphological features of this area may influence the availability of Caspian seal food resources."

In January 2023, similar to January 2021, seals moved away from the northern coast of the Caspian Sea (refer to Figure 5.2.3 J, K) and approached the edge of the ice fields where dense ice suitable for animal stranding was observed at that time (refer to Figure 5.2.3 B, C). Meanwhile, in January 2024, the highest number of animal locations was further north compared to January 2021 and 2023, and key sites during this period were also restricted to areas of solid ice with the greatest available thickness at that time (10-30 cm; refer to Figure 5.2.3 D).

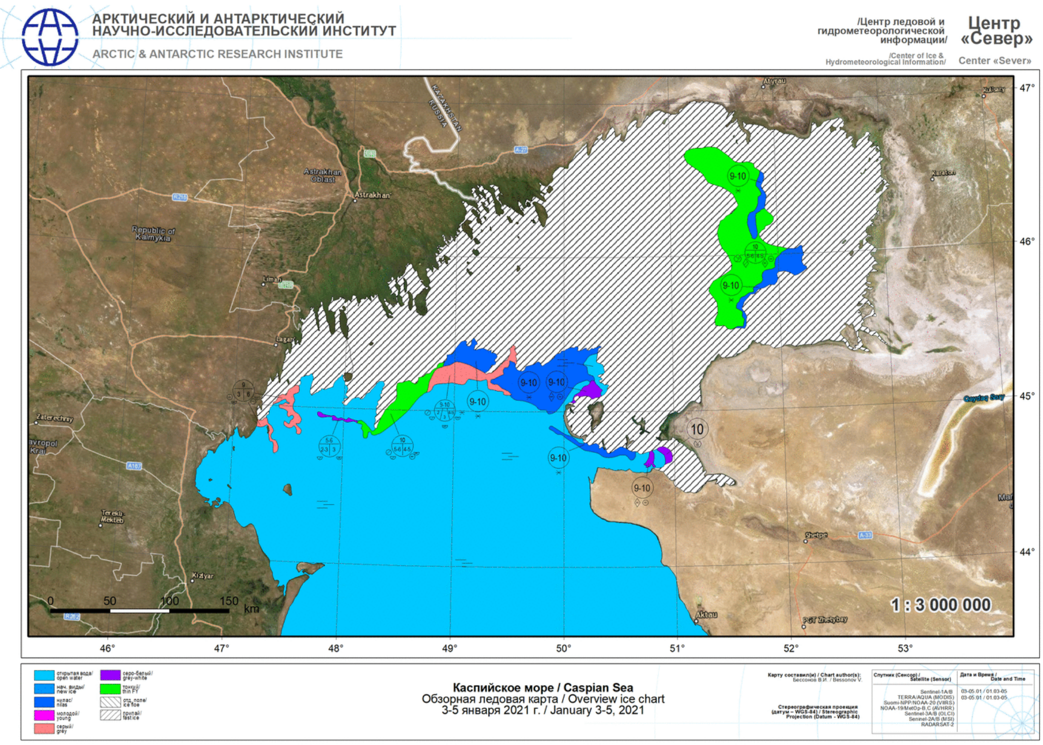
It is worth noting that in the winter of 2023-2024, ice covered most of the northern Caspian Sea water area, similar to 2020-2021 and 2022-2023, but generally exhibited lower thickness compared to the aforementioned years. The most prevalent ice types in January and February 2024, according to data from FGBU SIC Planeta, were nilas (up to 10 cm thick), gray ice (10-15 cm thick), and gray-white ice (15-30 cm thick). For February 2024, the key animal areas were noted to be located north of Seal Island, where, according to data from FGBU SIC Planeta, the boundary of ice fields with ice thickness of 10-30 cm was situated."

For animals tagged in 2023, as well as those tagged in 2020 and 2022, a more active use of the middle part of the Caspian Sea is characteristic compared to the 2019 tagging. Additionally, in these years, there is noted active use of the eastern coast of the Caspian Sea, whereas for the 2019 tagging, seals more often used the water area near the western coast of the Caspian Sea for movements.

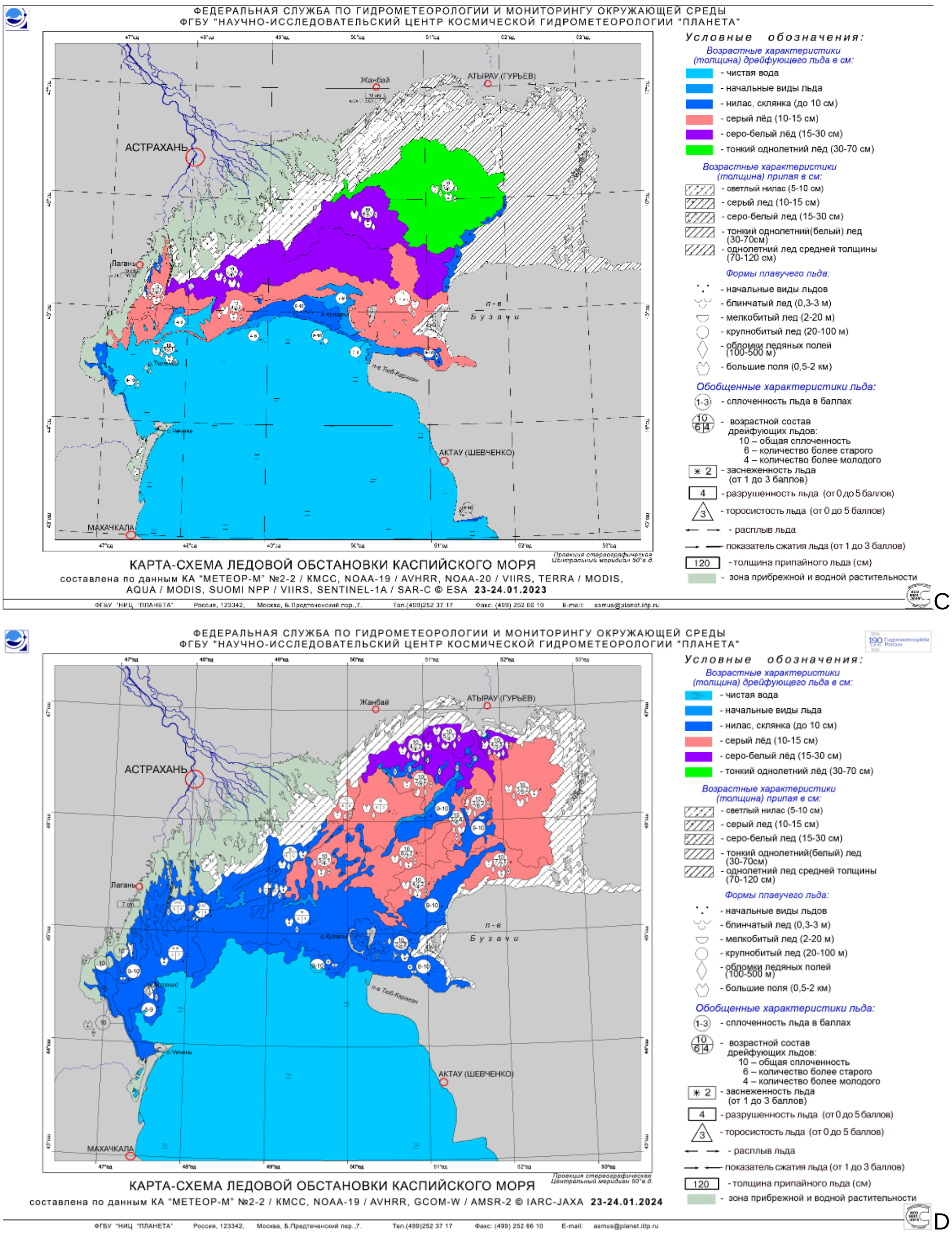
The utilization of coastal water areas for movements by animals (especially the eastern part of the sea along the coast of Kazakhstan) was previously identified in the literature as a "migration corridor" for autumn movements of animals [Dmitrieva et al., 2016]. It appears that the "migration corridor" noted earlier in the literature exists not only in the fall but also during the winter season for intraseasonal migrations of animals, possibly associated with the concentration of seal food sources. Continuing tagging efforts to expand the sample size, as well as tagging in other seasons, seems promising to compile a comprehensive picture of Caspian seal migrations throughout the year.



A



B



Sources: http://www.aari.ru, http://planet.rssi.ru

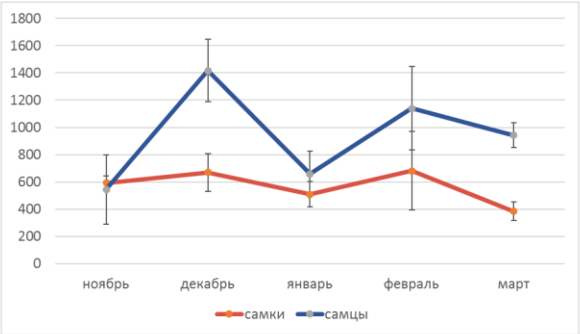
A - February 9-11, 2020; B - January 3-5, 2021; C - January 23-24, 2023; D - January 23-24, 2024

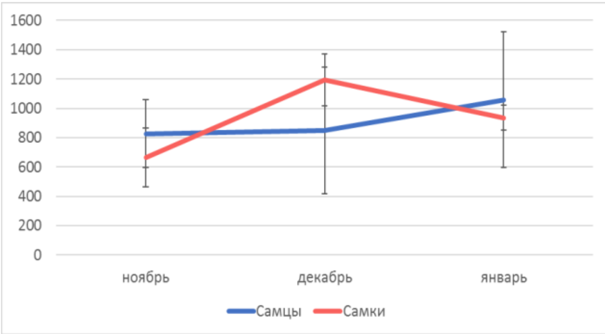
Figure 5.2.3 Ice Charts of the Northern Caspian Sea

Comparisons between 2022 and 2023 and tagging in 2019 and 2020 can only be made concerning female movement activity, as only females were tagged in 2022 and 2023.

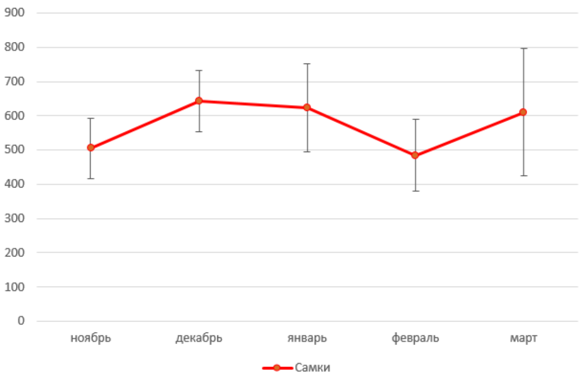
There are both similarities and differences in movement activity in the tagging years 2019, 2020, 2022, and 2023 (refer to Figure 5.2.4), but no clear patterns emerge. The activity of females in all tagging years is similar in terms of an increase in distance traveled from November to December, followed by a decrease in January. Similar patterns are also observed between the 2022 and 2023 tagging years, with mean values in February decreasing further and being lower than in January (refer to

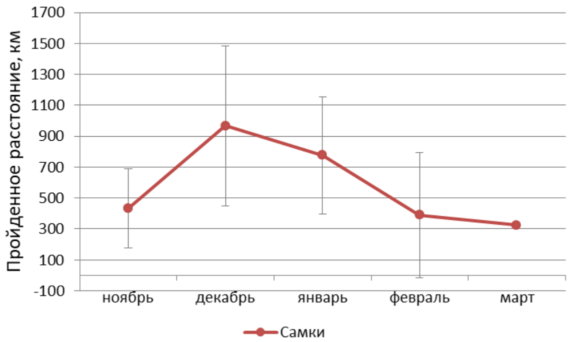
Figure 5.2.4 C, D). The values for March are not comparable because only one tag was still active in March 2024, and the data itself was only available for the beginning of the month (tracking was completed on March 10, 2024). Females traveled longer distances in 2020 than in other years.





A **B**





C **D**

A - labeled in 2019, B - labeled in 2020, C - labeled in 2022, D - labeled in 2023.

The X-axis represents the month, while the Y-axis represents the distance traveled (km ± SD).

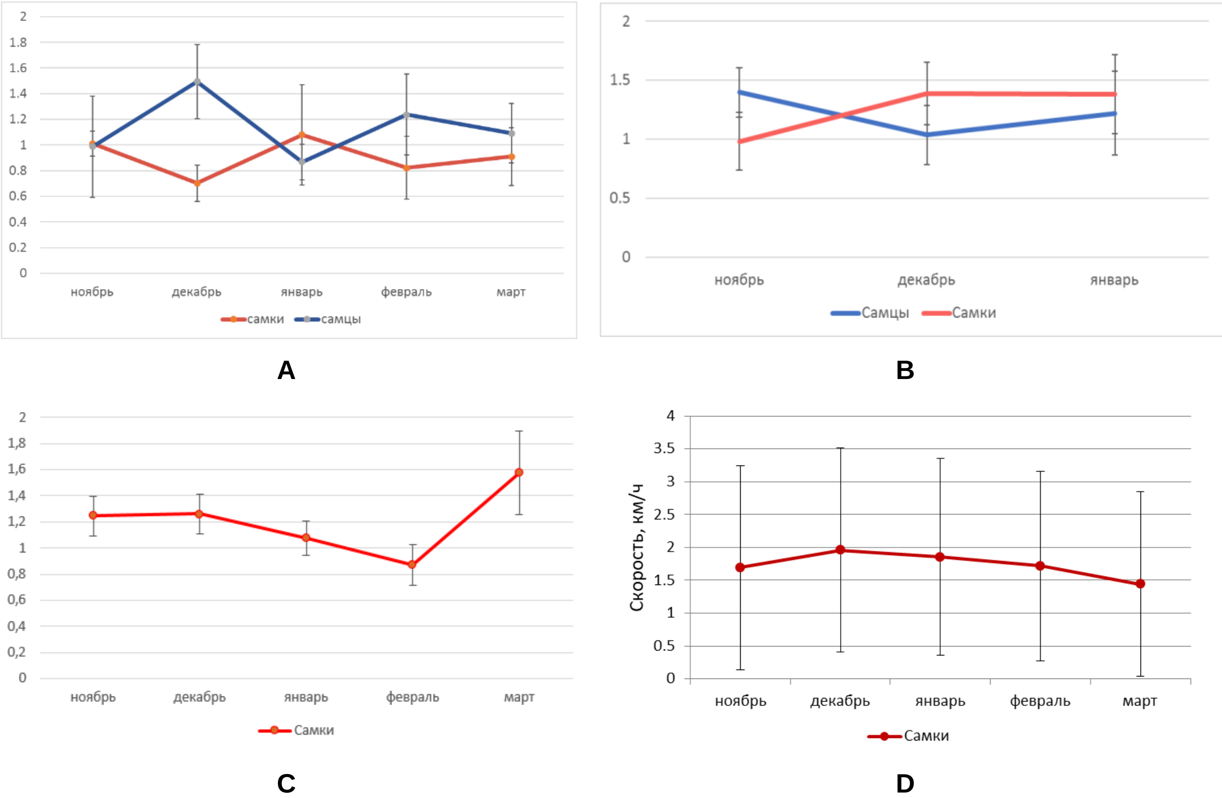
Figure 5.2.4 Activity of Seal Movements

The dynamics of speed rates differ for all four tagging years, with the only similarity noted being a decrease in speed rates in February compared to January values for animals tagged in 2019, 2022, and 2023 (refer to Figure 5.2.5). The lack of traceable patterns may be due to data quality for some months - in 2022, many tags did not transmit any locations, which affects sample size and quality. To discern a pattern in changes in the above parameters, a larger temporal coverage of tracking is needed, which could be achieved by tagging animals in different seasons, not just in the fall.

According to literature data, Caspian seals undergo meridional migrations during the spring and fall seasons (Sokolsky, 1998; Ivanov, Sokolsky, 2000; Dmitrieva et al., 2016). Data from females tagged in the years 2019, 2020, 2022, and 2023 were analyzed relative to their location at a specific latitude (refer to Figure 5.3.6).

In 2019, the movements of females mainly occurred within the northern Caspian Sea, with few venturing into the middle Caspian Sea. Instances where animals were recorded in the middle Caspian Sea only took place within the 43rd parallel (refer to Figure 5.2.6 A). In 2020, out of the five females, three spent a considerable time in the middle Caspian Sea, while one female (No. 207919) ventured as far as the southern Caspian (refer to Figure 5.2.6 B). In 2022, 11 out of 18 females migrated to the middle Caspian, with most of them spending more than a month in this region (refer to Figure 5.2.6 C). In 2023, 8 out of 13 tagged individuals moved to the middle Caspian (refer to Figure 5.2.6 D).

In 2020 and 2022, some individuals were tagged entering the southern Caspian beyond the 40th parallel (for December 2020, female No.197087; for January 2022, female No.238200). Similarly, in 2023, as well as in 2020 and 2022, some individuals migrated to the southern Caspian (tags No.253213 and No.253220).



A - labeled in 2019, B - labeled in 2020, C - labeled in 2022, D - labeled in 2023

Figure 5.2.5 Speed Indices (for 2019 and 2020) and Absolute Value of Speed (for 2022 and 2023), km/h

The latitudes of the 2019 locations differ significantly from those of the 2020, 2022, and 2023 tagging years. The tagging data for 2020, 2022, and 2023 show similarities in individual movements toward the southern Caspian. However, the data for 2020 and 2022 also exhibit similarities in the total duration spent by animals in the middle Caspian (exceeding a month for each animal recorded in both the middle and southern Caspian). In contrast, animals tagged in 2023, recorded in both the middle and southern Caspian, spent no more than a month in these regions of the water area.

The fall-winter season of 2019 exhibited distinct meteorological conditions compared to the corresponding seasons in 2020, 2022, and 2023, which experienced more "typical" winter weather patterns. The observed differences in meridional migrations may be attributed to the distribution of seals' food sources influenced by meteorological conditions.

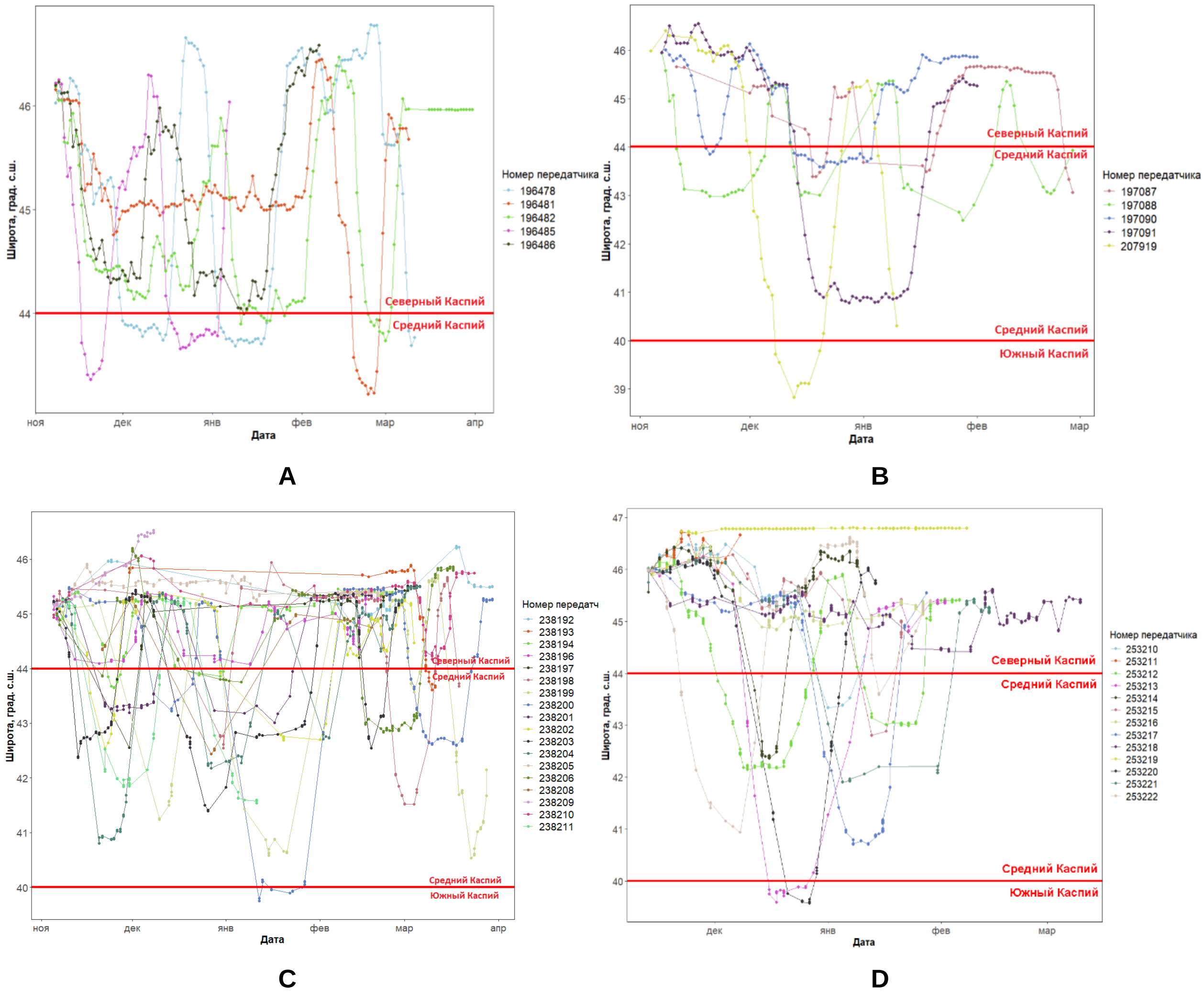
Comparison of the dynamics of the indicator of animals' distance from the shore is depicted in Figure 5.2.7.

In 2019, animals were farther from the shore in November-January compared to 2020 but closer than in 2022. Tagging data from 2023 also indicated an increase in shore distance indices from November to January, with median values for February lower than for January. Overall, animals tagged in 2019 were closer to the shore in February than those in 2020, 2022, and 2023. For the 2020 and 2022 tagging years, the maximum median values of animals' distance from shore were observed in February.

These variations across different years may also be attributed to the peculiarities of ice formation conditions. In 2019, the ice cover primarily formed near the coastline, whereas in 2020 and 2022, ice covered nearly the entire northern part of the Caspian Sea, possibly contributing to the elevated

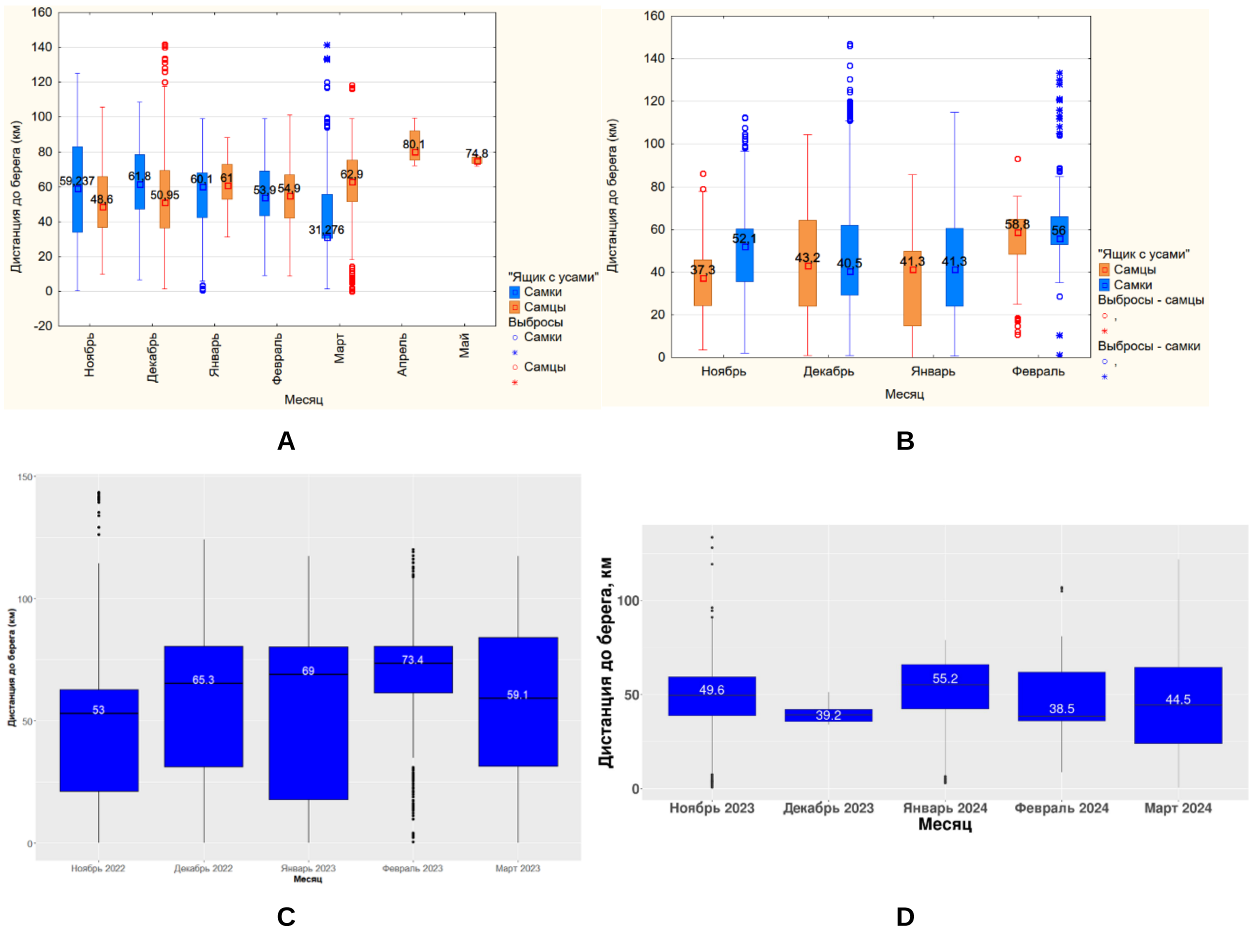
February values of animals' distance from the coast. In 2023, despite the extensive ice cover across most of the northern Caspian Sea, the southern distribution was predominantly occupied by nilas with a thickness of less than 10 cm. Areas with maximum thickness of 10-30 cm were located north of the Seal Islands and, consequently, closer to the northern coast of the Caspian Sea.

It's worth noting that for all the periods under consideration, there is a significant overlap in monthly data, indicating the absence of reliable differences in the remoteness index by month. Additionally, in all cases, locations in close proximity to the shoreline were recorded, reflecting animal movements along the coastline.



A - tagged in 2019, B - tagged in 2020, C - tagged in 2022, D - tagged in 2023

Figure 5.2.6 Graph of Latitude Variation in Signals (1 point per day) Received from Females

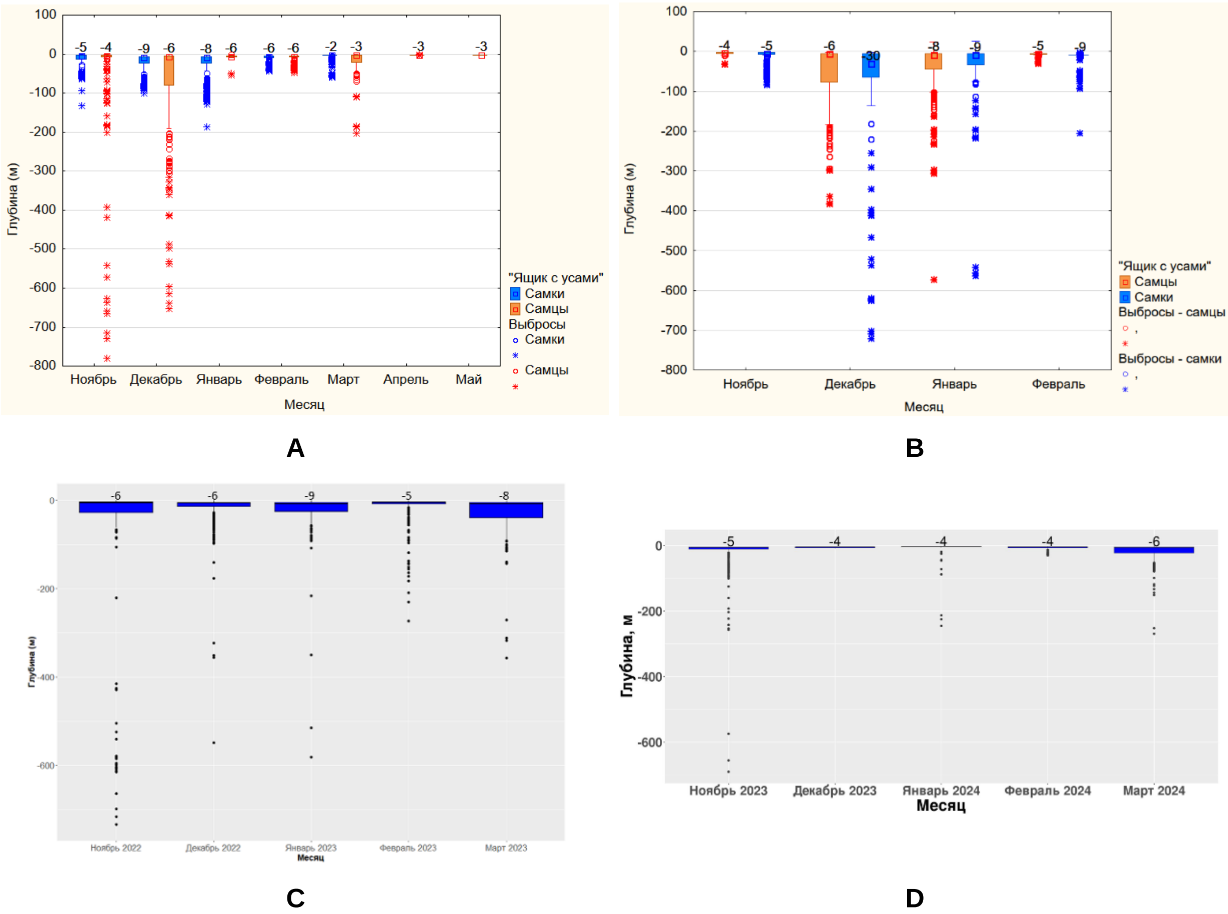


A - tagged in 2019, B - tagged in 2020, C - tagged in 2022, D - tagged in 2023

Figure 5.2.7 Distance to shoreline

Throughout all tracking periods, the majority of locations were recorded within the 150-meter isobath (Figure 5.2.8). This finding corroborates other studies on the distribution of Caspian seals in relation to bathymetric indices, as more than 80% of all seal dives traced from 2009 to 2012 occurred at depths less than 15 meters [Dmitrieva et al., 2016].

The low values of bathymetric indices in seal locations can be attributed primarily to the presence of animals in the shallow northern Caspian Sea for most of the tracking period. Additionally, these indicators also reflect the movement of animals along the coastal areas of the water. In 2023, seals were rarely recorded in areas with depths exceeding 500 meters, with only a few instances of seals in water areas with depths ranging from 200 to 500 meters. This pattern arises from the fact that seals venture into deep-water areas during their movements in the Caspian Sea but do not remain there for extended periods.

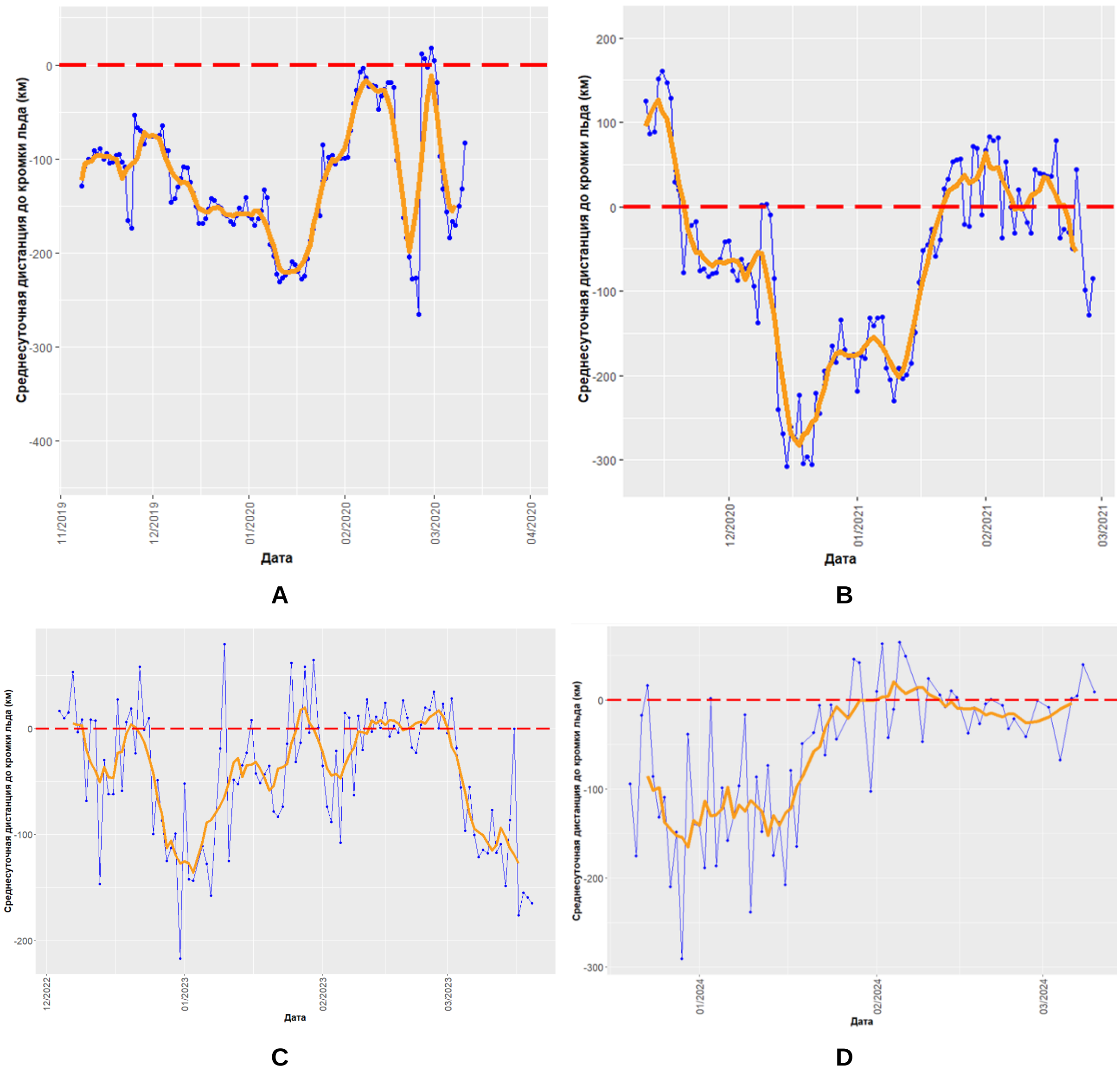


A- tagging 2019; B- tagging 2020; C- tagging 2022; D- tagging 2023

Figure 5.2.8 Habitat depths by month for females and males

The Caspian seal is a pagophilic species whose distribution is closely related to ice conditions. The movements of sexually mature animals in the fall-winter period are primarily determined by the timing of ice cover formation and breakup, ice cohesion and thickness, and the position of the ice edge [Ivanov and Sokolsky, 2000].

Due to the impossibility of comparing ice parameters in seal locations in 2022-2023 with those in 2019 and 2020 (due to the absence of ice cover metadata from FGBU "AARI" since 2022), we compared the distance of seal locations from the ice edge. A 7-day moving average (Figure 5.2.9) was used to compare data on the position of seals relative to the ice edge.



A - Tagging 2019; B - Tagging 2020; C - Tagging 2022; D - Tagging 2023.

The red dashed line represents the position of the ice edge.

Positive values indicate being within the ice field, while negative values indicate being on open water.

The blue line represents the average values for each date.

The orange line represents the 7-day moving average.

Figure 5.2.8 Distance to Ice Edge for Females

The values of the ice edge distance indicators for all tracking periods are similar. With the exception of November 2020, during all tracking periods, animals were almost always either on open water or near the ice edge, without venturing deep into the ice field. The presence of animals in the depth of ice field spreading in November 2020 is probably explained by the fact that the initial ice types formed in November 2020 did not hinder the animals' movement inside the ice fields.

The dynamics of animal movements relative to the ice edge by months are also similar for all tracking periods. In December and mid-January, animals moved away from the ice edge, and in February, during the seal breeding period, they were in close proximity to the ice spreading boundary. Similarities are noted for March 2020 and 2023, when animals, after a long stay near the ice edge, then moved away from it for considerable distances to ice-free areas. It is worth noting the similarity of distance to the ice edge from late January to early March for tagging in 2022 and 2023. During these periods, females were in close proximity to the ice edge. This similarity can be explained by the fact that in 2023, as in 2022, the absolute majority of tagged individuals were sexually mature females that moved to the ice edge in late January to February to pup and mate.

The obtained data, as well as comparison of the 2023 tagging data with the tagging data of 2019, 2020, and 2022, form an initial idea about the migration behavior of the Caspian seal in the autumn-winter period. To compile a complete picture of Caspian seal movements, it is necessary to further tag animals, including in other seasons, which will significantly increase the sample size, verify the previously obtained data, and identify patterns in the movements of seals not only in the fall-winter period but also in other seasons of the year.

5.3. Virological studies

In our virological analysis, we found no evidence of nucleic acids from pathogens such as influenza (both A and B viruses), coronavirus, paramyxovirus (including carnivore morbillivirus), hepivirus (hepatitis E), poxvirus, lyssavirus, or retrovirus infections in samples taken from Caspian seals. However, PCR products matching the expected sizes of adenovirus and herpesvirus gene fragments were detected in nasal wash samples collected in 2023 from animals exhibiting signs of respiratory infection. These findings suggest the presence of mixed respiratory infections among the studied group, caused by adenoviruses and herpesviruses of seal serotype 2. Given the prominent role of viruses in infectious pathology, further research is warranted.

In the virome of Caspian seals, we identified two major groups comprising different families of viruses. The first group includes families such as Circoviridae and Parvoviridae, which are associated with the aquatic ecosystem. These viruses primarily infect various organisms in the marine environment of the Caspian Sea, such as invertebrates for circoviruses and insects and crustaceans for densoviruses of the Parvoviridae family. Notably, this group constitutes 72% of the total Caspian seal virome, suggesting a possible dietary origin. The second group encompasses mammalian viruses, including Herpesviridae, Papillomaviridae, Caliciviridae, Anelloviridae, Adenoviridae, Orthomyxoviridae, and Paramyxoviridae. Viruses within this group have the potential to cause various pathologies in mammals or may remain asymptomatic.

Taxa representing 22 types of bacteria were discovered in the microbiome of Caspian seals, with five types predominating in the dataset: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Fusobacteria. We observed similarities in the microbiome profile of respiratory, alimentary, and urogenital tract samples collected from animals between 2020 and 2023. Additionally, we noted variations in the percentages of bacterial families depending on the type of sample collected from Caspian seals.This study underscores the importance of continuous monitoring of the microbiome of Caspian seals to detect the introduction of clinically significant bacterial pathogens into their population. Overall, the findings of this study lay a solid foundation for future research and enhance our understanding of host-microbe interactions in the Caspian seal population.

The obtained serological data complement the results of PCR screening for the presence of influenza A virus in the samples of seals that were collected from both the Kazakh and Russian parts of the sea at the end of 2022. This indirectly indicates the involvement of the pathogen in an outbreak of epizootic influenza infection among seals, which was accompanied by high mortality rates. Antibodies to CDV were not detected in the sera of seals collected in 2020, but they were present in up to 28% of the samples collected in 2022. Additionally, one serum obtained in 2019 and two samples from 2023 were also positive for antibodies to the carnivore plague virus. These findings suggest a recent outbreak of usazan infections in these animal populations.

According to the results of studies conducted on 22 samples of seal feces using the Darling and Scherbovich methods, no helminth eggs, including those of the helminth Anisakis chupakovi, were found. The absence of helminth eggs in the feces of seals may indicate either a small sample size of the material under study or the absence of infested seals during the study period.

5.4. Microbiological studies

The alpha-diversity indicators of the nasal microbiome of the Caspian seal indicate moderate conservatism and low stability of this community, which is typical for marine predators. Such animals usually harbor a small number of microorganism species in their nasal mucosa, making it vulnerable to pathogenic microbes. This vulnerability is particularly notable in individuals No.1 and No.17. While the structure of the nasal microbiome at the phylum level closely resembles that of humans [Ramakrishnan et al., 2018] and canines [Horsman et al., 2023], it includes some very specific genera, such as Coenonia (more commonly found in waterfowl [Vandamme et al., 1999]) and Thioclava. Since this is the first study of its kind conducted on seals, there are no direct references for comparison. Notably, typical genera found in other animals, such as Staphylococcus, Streptococcus, Oligella, and Bergiella, were not encountered.

The alpha diversity indices of the microbiome of the rectal mucosa exhibit typical values, with a high Shannon index and a rather high Simpson index indicating an undisturbed community with high stability. The hindgut community structure of harbor seals has been well described in previous studies on Phoca vitulina [Numberger et al., 2016; Pacheco-Sandoval et al., 2019], Mirounga leonina, and Hydrurga leptonyx [Nelson et al., 2012]. In all cases, it was represented by four main phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria. In analyzing the results of the Caspian seal study, six phyla mentioned above, as well as Actinobacteria and Epsilonbacteraeota, were isolated. The appearance of Epsilonbacteraeota was previously part of Proteobacteria, so its presence is technical and does not alter the findings. However, the dominance of Actinobacteria can be considered a unique feature of the Caspian seal. Although the dominance of Actinobacteria has also been shown in Neophocaena phocaenoides [McLaughlin et al., 2012] and Cystophora cristata, it was not to the same extent as observed in the Caspian seal.

Of particular interest is the almost complete absence of representatives of the family Enterobacteriaceae, which typically hold significant clinical importance. This sets the Caspian seal apart sharply from both terrestrial carnivores [Pilla R and Suchodolski, 2020] and marine mammals [Bik et al., 2016; Ley, R. E. et al., 2018; Wan et al., 2018; Sehnal et al., 2021]. The low proportion of representatives of Lactobacillales, including Lactobacillus and Streptococcus, also deserves attention.

The alpha diversity indices of the microbiome of the urogenital mucosa exhibit very high values, with Shannon and Simpson indices higher than those in the rectal microbiome. However, this area is still poorly understood, so no reference for seals is available. At the phylum level, the microbiome shares many similarities with the vaginal microbiome of other animals (Barba et al., 2012). Representatives of Enterobacteriaceae and Lactobacillus are almost entirely absent from the urogenital mucosa.

Beta diversity analysis suggests that the colonization pathways of the intestinal and vaginal mucosa may be similar, with the enterovaginal pathway being the most likely route. This is supported by their close positions in the principal coordinates analysis (Figure 5.4.1) and the total number of OTUs (Figure 5.4.2). Despite the high heterogeneity of the samples, the nasal community differs significantly from the urogenital and rectal communities (PERMANOVA, $p < 0.05$)

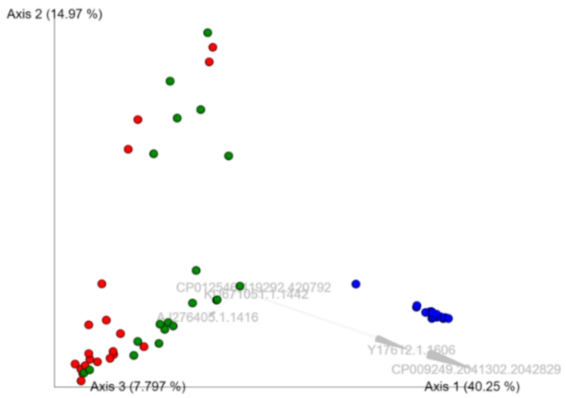


Figure 5.4.1 **Beta Diversity Analysis of the rectal (red marker), vaginal (green marker), and nasal (blue marker) microbiomes of Caspian seals**

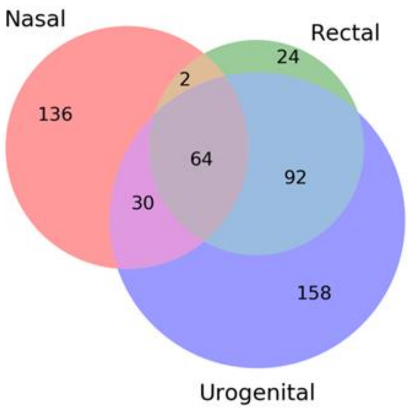


Figure 5.4.2 Venn diagram illustrating the distribution of OTUs between mucosae

The following list of facultative pathogens is assumed for seals [Siebert et al., 2001; Sonne et al., 2020]: Aeromonas spp., Bacteroides fragilis, Clostridium perfringens, Erysipelothrix rhusiopathiae, Escherichia coli var. haemolytica, Klebsiella spp., P. aeruginosa, Salmonella spp., Serratia spp., Staphylococcus aureus, Staphylococcus intermedius, α- and β-haemolytic streptococci, Vibrio anguillarum. It has been indicated that streptococci are the main causative agents of diseases, at least for ringed seals [Bergman, 2007].

In the Caspian seal, streptococci are not among the characteristic genera, which sharply distinguishes it from most animals, including pinnipeds. Also interesting is the absence of Escherichia coli. Other facultative pathogens of the Enterobacteriaceae family are also practically absent.

Representatives of Pasteurellaceae and Campylobacteriaceae are traditionally considered facultative pathogens of carnivores and causative agents of wound infections in humans. In Caspian seals, representatives of these families are obligate symbionts and should not be considered pathogenic.

The microbiome of the Caspian seal contains microorganisms that were previously isolated in pinniped animals (seals, including those living in the other hemisphere): Corynebacterium (phocae), Campylobacter sp., Porphyromonas sp., Guggenheimella sp., Bergeyella sp., Bisgaardia (hudsonensis).

The 2023 study allowed for the correction of the 2019 and 2022 project data. The microbiome structure was significantly expanded, and alpha diversity indicators were calculated. The sample expansion enabled the exclusion of genera such as Peptoniphilus, Atopobacter, Clostridium, Arcanobacterium, Salinicoccus, Coenonia, Gemella, and Capnocytophaga from the list of typical representatives of both urogenital and rectal microbiota. While these microbes are widely represented in Caspian seals, they are found in less than 80% of individuals. Cardiobacteriaceae sp., Ornithobacterium, and Salimicrobium are found only in single individuals and cannot characterize the species as a whole. In contrast, Campylobacter and Fusobacterium should be included in the list of typical representatives of the urogenital microbiota. Detailed annotation of all sequences obtained allowed us to additionally identify two undescribed microorganisms, as well as six microorganisms that occur only in pinnipeds.

5.5. Toxicological studies

Throughout the research period (2019-2023), mercury content in the coat was determined in 115 Caspian seal individuals aged from a few months to 9 years. Mercury concentrations in the hair coat ranged from 258 µg/kg to 10285 µg/kg. Values between 1000 and 3000 µg/kg were the most common (53% of individuals). (Table 5.5-1).

Table 5.5-1 Frequency distribution of mercury concentrations in Caspian seal vibrissae

Hg in wool, µg/kg	Frequency of occurrence, number of individuals	Cumulative frequency of occurrence, number of individuals	Percentage of total number	Cumulative percentage
0–1000	13	13	11	11
1000–2000	29	42	35	36
2000–3000	32	74	28	64
3000–4000	17	91	15	79
4000–5000	11	102	10	89
5000–6000	4	106	3	92
6000–7000	2	108	2	94
7000–8000	3	111	3	97
8000–9000	2	113	2	99
9000–10000	1	115	0.5	99.5
10000-11000	1	115	0.5	100

During the period from 2019 to 2023, mercury content in the vibrissae was determined in 102 Caspian seal individuals. The minimum value recorded was 954 µg/kg, while the maximum value reached 12957 µg/kg. Concentrations between 2000 and 4000 µg were the most frequent, observed in 66 individuals, which accounted for 64% of the total sample (Table 5.5-2).

Table 5.5-1 Frequency distribution of mercury concentrations in Caspian seal vibrissae

Hg in wool, µg/kg	Frequency of occurrence, number of individuals	Cumulative frequency of occurrence, number of individuals	Percentage of total number	Cumulative percentage
0–1000	1	1	1	1
1000–2000	13	14	13	14
2000–3000	30	44	29	43
3000–4000	36	80	35	78
4000–5000	10	90	9	87
5000–6000	7	97	7	94
6000–7000	2	99	2	96
7000–8000	2	101	2	98
8000–9000	0	101	0	98
9000–10000	0	101	0	98
10000–11000	0	101	0	98
11000–12000	0	101	0	98
12000–13000	2	103	2	100

Blood mercury concentrations were measured in 64 Caspian seal individuals in 2020, 2022, and 2023, ranging from 29 to 350 µg/L of crude blood. Concentrations between 100-150 µg/L were most common, observed in 54 individuals, representing 84% of the total sample (Table 5.5-3).

Table 5.5-3 Frequency distribution of mercury concentrations in Caspian seal blood

Hg in wool, µg/L	Frequency of occurrence, number of individuals	Cumulative frequency of occurrence, number of individuals	Percentage of total number	Cumulative percentage
0–50	1	1	2	2
50–100	33	34	52	54
100–150	22	56	34	88
150–200	5	61	6	94
200–250	1	62	2	96
250–300	1	63	2	98
300–350	1	64	2	100

No significant differences were found in mercury concentrations in hair, vibrissae, and blood among seals studied in different years (K-W (3, n = 115) H = 5.43. p = 0.142; K-W (2, n = 103) H = 0.59, p = 0.746) (Figures 5.5.1-5.5.3). The lack of differences suggests that biogeochemical conditions affecting mercury migration through the trophic networks of the Caspian Sea remain stable from year to year.

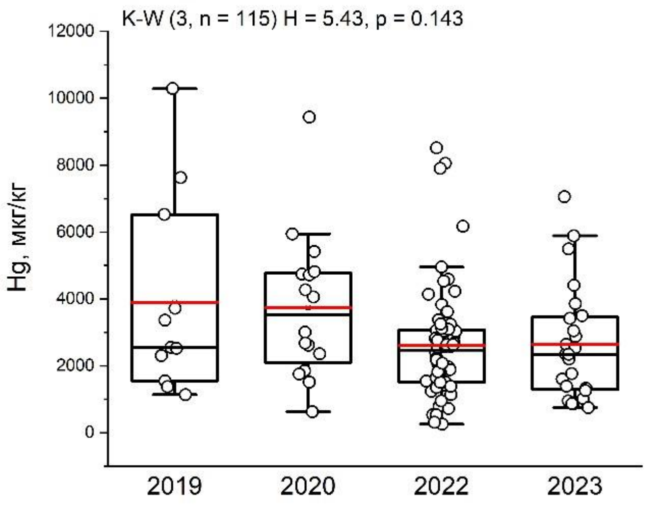


Figure 5.5.1 Mercury content in Caspian seal fur

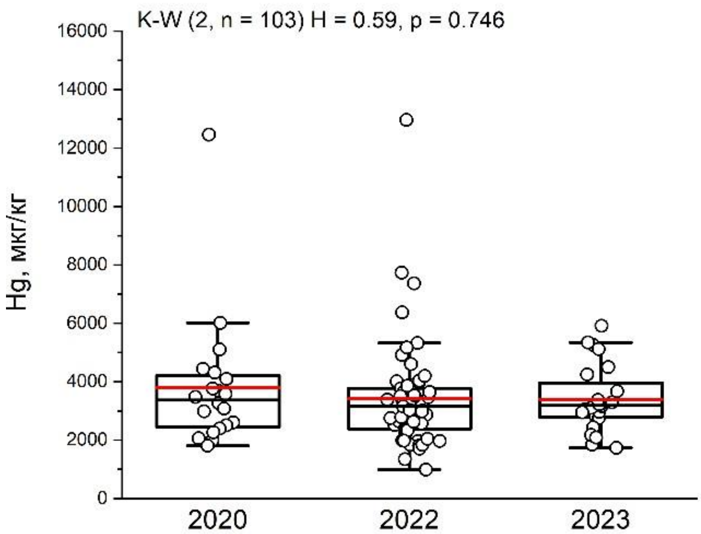


Figure 5.5.2 Mercury content in Caspian seal blood in different years of studies

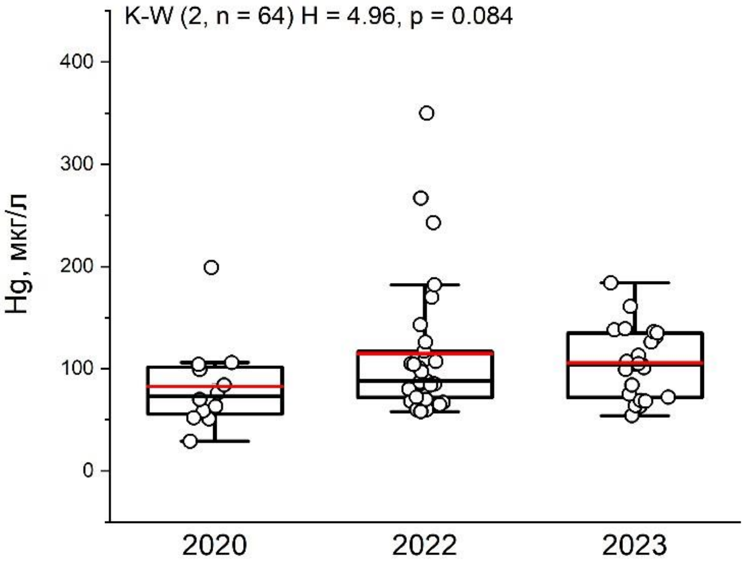


Figure 5.5.3 Mercury content in Caspian seal blood across various years of study

For detailed insights into the comparison of mercury concentrations in biomaterials between male and female Caspian seals, as well as correlation analyses, refer to Tables 5.5-4 and 5.5-5

Table 5.5-2 Results of pairwise comparison using the Mann-Whitney U test, along with Spearman correlation coefficients linking mercury concentrations in biomaterials with the length, weight, and age of seals across different years of the study

Years of study	2019	2020	2022	2023	All years
Mercury in wool					
Differences between males and females	U = 7 Z = 1.5	U = 21 Z = 0.32	U = 361 Z = 1.3	U = 16 Z = 1.8	U = 1310 Z = 0.9
Length	**0.73**	-0.02	**0.37**	**0.78**	**0.68**
Weight	**0.77**	0.30	0.27	**0.79**	**0.69**
Age	**0.76**	0.01	**0.34**	**0.74**	**0.36**
Mercury in vibrissae					
Differences between males and females	- -	U = 18 Z = 0.71	U = 471 Z = 1.9	U = 20 Z = 1.5	U = 852 Z = 1.9
Length	-	**0.54**	-0.17	**0.52**	0.12
Weight	-	0.17	-0.17	**0.48**	0.23
Age	-	0.03	0.15	**0.44**	**0.23**
Mercury in the blood					
Differences between males and females	- -	U = 15 Z = 0.40	U = 48 Z = 0.06	U = 24 Z = 1.1	U =323 Z = 0.43
Length	-	0.31	**-0.42**	-0.22	-0.22
Weight	-	-0.10	**-0.48**	-0.30	**-0.30**
Age	-	-0.04	-0.19	-0.27	-0.19

***Bold font** indicates reliable values of the correlation coefficient at the significance level of p < 0.05*

Table 5.5-5 Spearman correlation coefficients (ρ) between mercury concentrations in Caspian seal biomaterials in different years of study

Years of study	2020	2022	2023	All years
Hg in wool/Hg in vibrissae	0.68	0.57	0.75	0.67
Hg in wool/ Hg in blood	0.51	0.33	0.11	0.26
Hg in vibrissae/Hg in blood	0.31	0.63	0.42	0.47

Mercury concentrations in Caspian seal fur are either higher or comparable to those found in the fur of other pinniped species inhabiting the Russian Federation (see Table 5.5-6).

Differences in mercury concentrations between Caspian seals and other pinniped species may be attributed to their preferred food items. The elevated mercury levels in Caspian seals compared to those in Baikal seals may be due to the higher mercury content in Caspian Sea fish (ranging from 60-670 µg/kg) compared to fish from Lake Baikal (20-30 µg/kg) [Pastukhov, 2011; Tabatabaie et al., 2011; Manavi and Mazumder, 2018].

Table 5.5-6 Mercury levels in the fur of different pinniped species

Species	Region	Age	Hg, µg/kg	Source
Mercury in wool				
Caspian seal Pusa Caspica	Caspian Sea	Adult and juvenile individuals	560–3500	Ikemoto at al., 2004
Sea hare Erignathus barbatus	Russia, White Sea	Adult and juvenile males and females	540–920	Medvedev et al., 1997
Ringed seal Phoca hispida hispida			2500–6600	
Pygmy seal Phoca largha	Russia, Sea of Japan	Juveniles	1520–6680	Trukhin and Kalinchuk, 2018
Baikal seal Phoca sibirica	Lake Baikal. Baikal	Adult and juvenile individuals	640–3740	Pastukhov et al. 2011
				Ikemoto at al., 2004
Northern fur seal Callorhinus ursinus	Alaska	Adult females	7840±1780	Beckmen et al., 2002
The harbor seal Phoca vitulina	Southwest U.S.	Adult males and females	210–19620	McHuron et al., 2012
	Southwest U.S.	Adult males and females	5230–144310	Peterson et al., 2016
	Southwest U.S.	Young puppies	2800–36900	Van Hoomissen et al., 2015
Steller sea lion Eumetopias jubatus	Alaska	Individuals aged 1-2 years	770–3950	Castellini et al., 2012
	Alaska	Adult females	5400-41020	Peterson et al., 2016
California sea lion	Southwest U.S.	Adult females	5100–21000	Peterson et al., 2016
Mercury in the blood				
The harbor seal Phoca vitulina	German coastline	Adult and juvenile males and females	808–2420	Damseaux et al., 2020
Long-finned seal Halichoerus grypus			370–1285	
The harbor seal Phoca vitulina	Scotland's coastline		122–945	
Long-finned seal Halichoerus grypus			179–544	

Analysis of mercury content in tissues of pinnipeds, whose diet is based on fish and large invertebrates, serves as an objective indicator not only of the intensity of metal input into aquatic ecosystems but also of the structure of hydrobiont communities [Brown et al., 2015; Cipro et al., 2017]. Studies of mercury accumulation in wool and vibrissae of pinnipeds are still scarce. Therefore, determining mercury accumulation levels in these tissues, as well as in the blood of the Caspian seal, is necessary when assessing the risk to the species' existence.

The results of the study of mercury concentration in the Caspian seal coat and limited literature data allow us to conclude that for the majority of seal species (including the Caspian seal), there are no intersex differences in mercury content in the body.

Comparable mercury concentrations between males and females in the studied biomaterials of the Caspian seal confirm that the diet of individuals of both sexes is the same [Badamshin, 1948; Vorozhtsov et al., 1972]. The absence of differences between individuals at different stages of sexual maturity indicates that changes in the hormonal background at various physiological states of the Caspian seal organism do not lead to changes in mercury concentration in the fur.

The weak correlation between mercury content in fur and age suggests that mercury accumulates in the body over an individual's lifespan, which is reflected in its content in wool immediately after molting. It is possible that the correlation between age and mercury content in organs (primarily kidneys and liver) may be stronger than that between age and mercury content in hair.

In 2023, a significant positive correlation was found between mercury content in vibrissae and body size. However, there was no such correlation in the samples from previous years. Apparently, the correlation observed in 2023 is due to an unrepresentative sample and does not reflect a real physiological effect.

The reference value for mercury concentration in human blood, above which neurotoxic effects manifest, is 200 µg/l [Clarkson and Magos, 2006]. Assuming that the functioning of the nervous system in pinnipeds is disturbed at the same concentration of mercury in blood, then 3 Caspian seals (5%) from the studied sample are in the zone of potential risk. This percentage is significantly lower than in a comparable sample of harbor seals from the west coast of the USA (n = 36), where blood mercury concentrations exceeded the reference value in 28% of individuals ([an Hoomissen et al., 2015].

Based on the results of these studies, it can be concluded that current levels of mercury input into the Caspian Sea ecosystem do not result in elevated mercury concentrations in biota.

5.6. Hormonal Studies

Hormonal studies were conducted using three different substrates: serum, hair, and vibrissae of the animals.

Most blood samples were collected from sexually mature animals. In this case, blood sampling took place in November, which, in adult females, corresponds to the gestation period. Progesterone levels in adult animals were found to be extremely high, significantly higher than during earlier collections in 2020 and 2021. In 2020 and 2021, almost all of the samples were collected from immature animals, which determined the low levels of sex hormones. In 2022, most of the animals were sexually mature, and judging from the hormonal profile, pregnant

5.6.1. Interannual Differences in Hormones among Different Age and Sex Groups

When analyzing inter-annual differences in hormone levels, animals were divided into two age categories: individuals aged 0 to 3 years were considered young animals, while individuals 4 years and older were considered adults. The number of individuals in different sex-age categories and pregnancy status for all years of the study is presented in Table 5.6-1.

Table 5.6-1 Number of Caspian seals in different sex-age categories and pregnancy status in the samples from 2019 to 2023.

Year of study	Number of young males	Number of adult males	Number of young females	Number of adult yearling females	Number of adult pregnant females
2019	2 alive, 1 dead	3	4	0	0
2020	4	2	5	2	0
2022	3 dead	2 alive, 17 dead	4 alive, 7 dead	8 alive, 0 dead	12 alive, 12 dead
2023	2	0	5	6	7

Comparisons were conducted in all groups except adult males due to their insufficient numbers in each year for statistical analysis.

Performance Comparison of Young Males

An interannual comparison of hormone levels in blood and hair was conducted for live young males from 2020 (n=4) and 2023 (n=2). No inter-annual differences were found when comparing blood and hair testosterone and hair progesterone concentrations in young males ($p>0.05$, Mann-Whitney test). In 2023, blood cortisol and progesterone concentrations were higher in young males than in individuals from the 2020 sample ($p<0.05$, Mann-Whitney test, Figures 5.6.1-5.6.2).

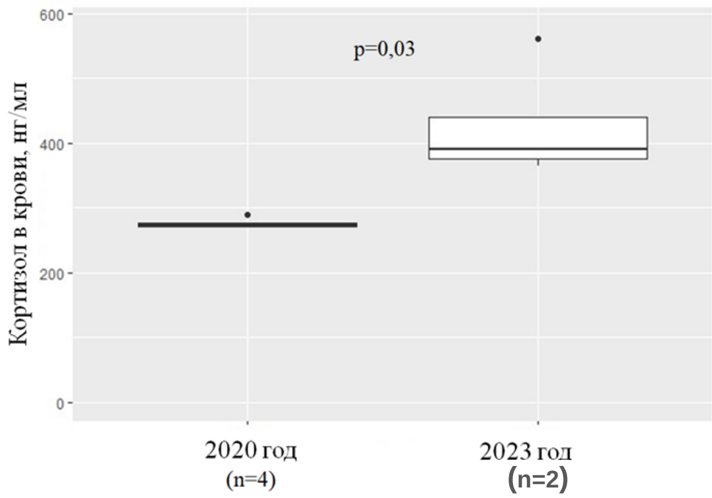


Figure 5.6.1 Blood cortisol concentrations in young males from the 2020 and 2023 samples

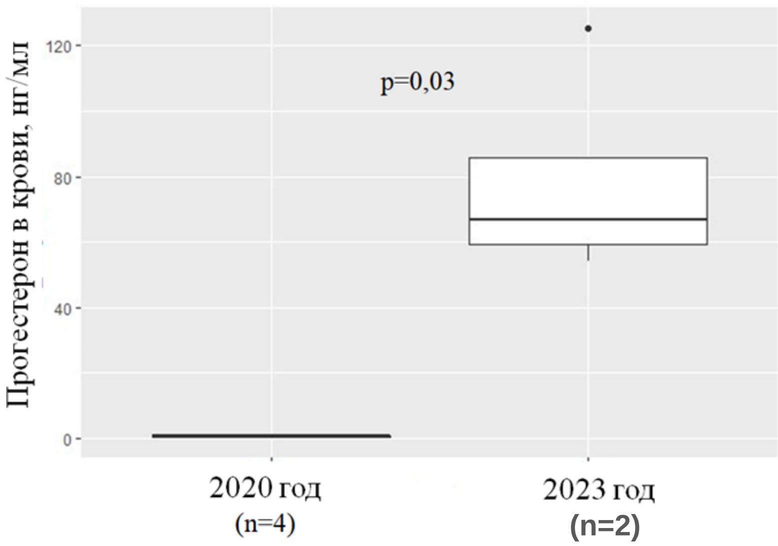


Figure 5.6.2 Progesterone concentrations in the blood of young males from the 2020 and 2023 samples

Comparison of the hormone levels of young females

An interannual comparison of blood and hair hormone levels was conducted in live young females from 2019 (n=4), 2020 (n=5), 2022 (n=4), and 2023 (n=5). No effect of the factor "year" on testosterone levels in blood and hair, and cortisol levels in blood (p>0.05, Kruskal-Wallis test) was detected. However, the influence of the factor "year" on the concentration of cortisol in hair was revealed (Kruskal-Wallis chi-squared = 12.532, df = 3, p=0.006, Kruskal-Wallis test) - in 2023, the concentration of cortisol in hair was higher compared to all previous years (p<0,05, (p<0.05, Mann-Whitney test), in 2022 the cortisol concentration was higher compared to 2019 (W = 0, p=0.03, Mann-Whitney test, Figure 5.6.3).

Blood progesterone concentration in young females was lower in 2020 compared to 2022 and 2023 (p<0.05, Mann-Whitney test), no differences were found between 2022 and 2023 (W = 9, p=0.41. Mann-Whitney test, Figure 5.6.4). The concentration of progesterone in the hair of young females was higher in 2023 compared to 2022 (W = 2, p= 0.04, Mann-Whitney test, Figure 5.6.5).

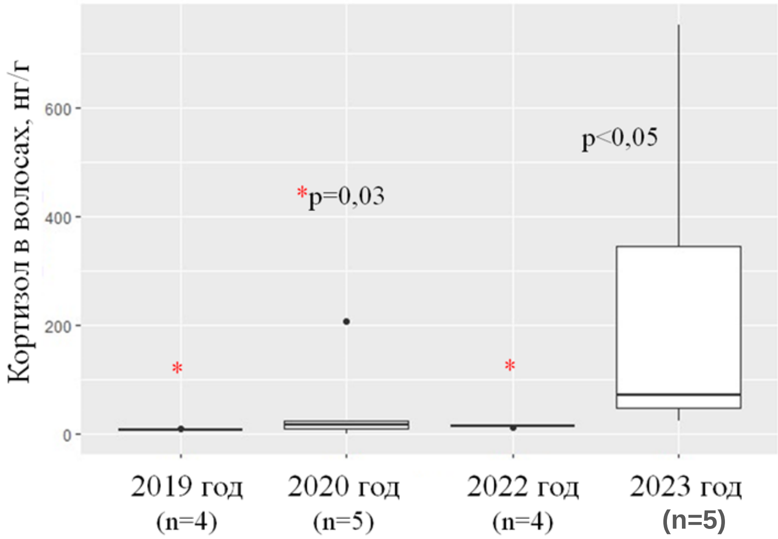


Figure 5.6.3 **Cortisol concentrations in the hair of young females from the 2020, 2020, and 2023 samples**

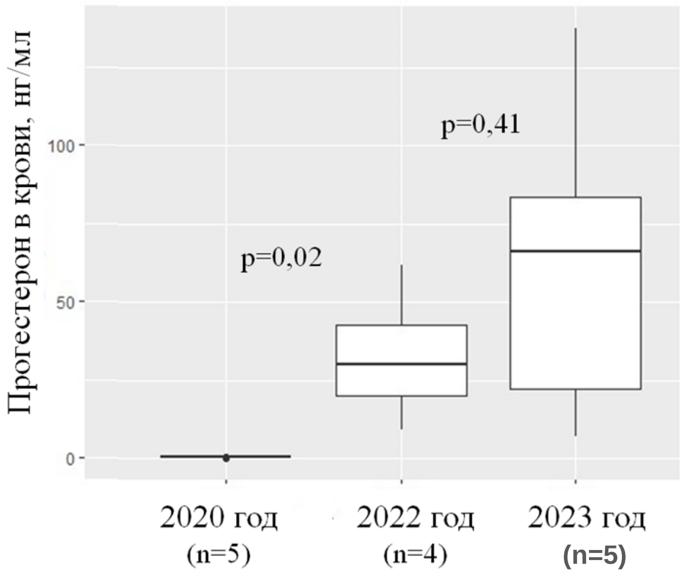


Figure 5.6.4 **Progesterone concentrations in the blood of young females from the 2020, 2022, and 2023 samples.**

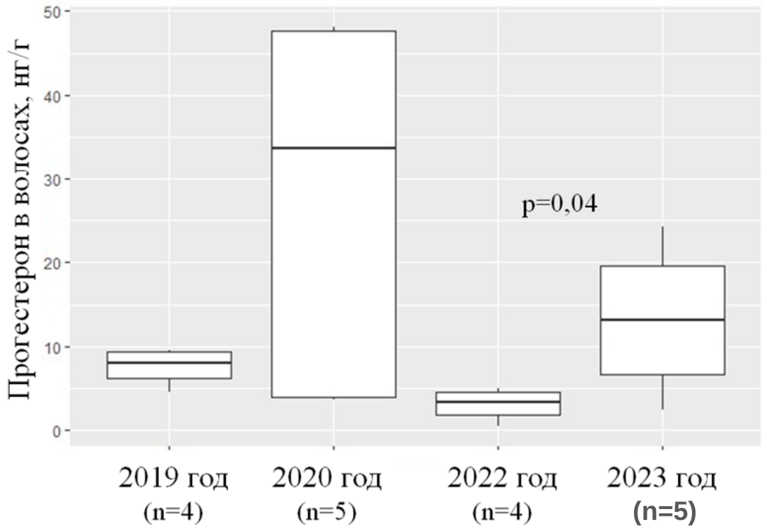


Figure 5.6.5 Progesterone concentration in the hair of young females

Comparison of the hormone levels of yawl females

An inter-annual comparison of hormone levels in the blood and hair of live yawl females from 2020 (n=2), 2022 (n=8), and 2023 (n=6) was conducted. No inter-annual differences were observed in cortisol and testosterone concentrations in the blood and hair of yawl females (p>0.05, Mann-Whitney test). However, blood progesterone concentrations were higher in 2022 than in 2020 (W = 0, p=0.04, Mann-Whitney test, see Figure 5.6.6); no differences were found between blood progesterone levels in 2022 and 2023 females (p>0.05, Mann-Whitney test).

In 2023, the concentration of progesterone in hair was higher than in 2022 (W = 6, p=0.02, Mann-Whitney test, see Figure 5.6.7); however, no differences were found between 2020 and 2022 (p>0.05, Mann-Whitney test).

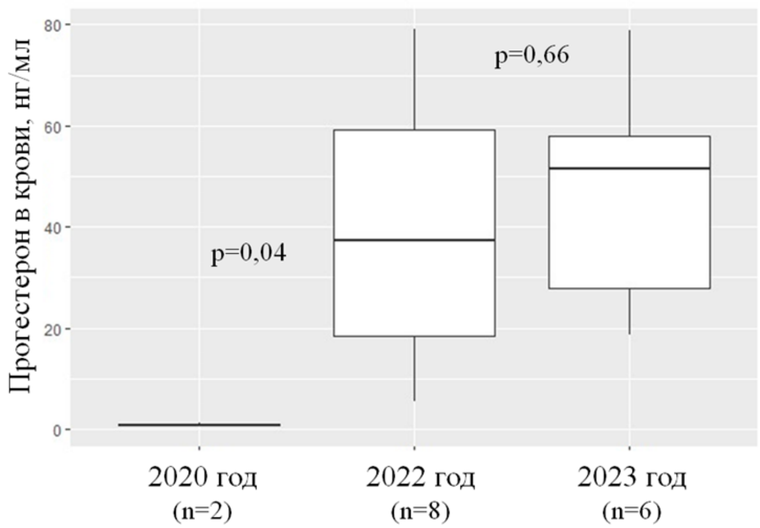


Figure 5.6.6 Progesterone concentrations in the blood of yawl females from the 2020, 2022, and 2023 samples.

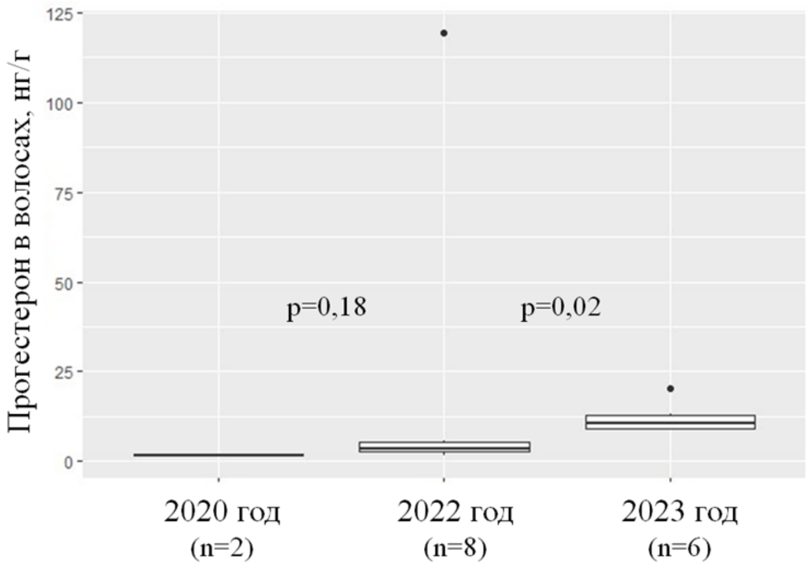


Figure 5.6.7 **Progesterone concentrations in hair of yawl females from the 2020, 2022 and 2023 samples**

Comparison of the hormone levels of pregnant females

An inter-annual comparison of hormone levels in the blood and hair of live pregnant females from 2022 (n=12) and 2023 (n=7) was conducted. No inter-annual differences were found in the concentrations of cortisol, testosterone, and progesterone in the blood of pregnant females (p>0.05, Mann-Whitney test). However, higher concentrations of cortisol (W = 18, p=0.045, Mann-Whitney test, see Figure 5.5.8) and progesterone in hair (W = 7, p<0,01, Mann-Whitney test Figure 5.5.9) were recorded compared to those of pregnant females in 2022.

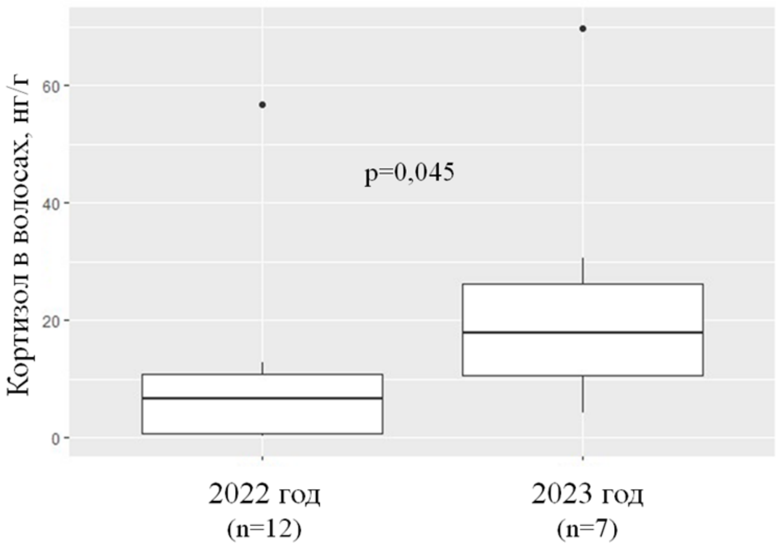


Figure 5.66.8 **Cortizol concentrations in the hair of pregnant females from the 2020, 2022, and 2023 samples.**

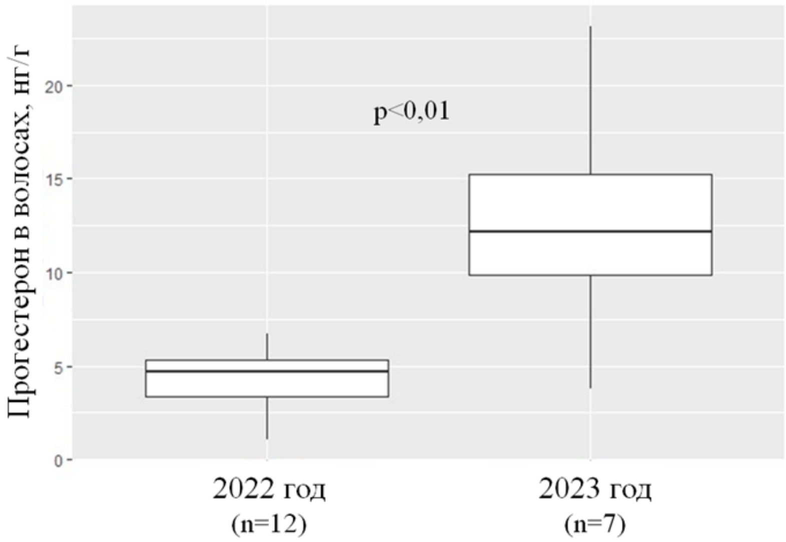


Figure 5.66.8 Progesterone concentrations in the hair of pregnant females from the 2020, 2022, and 2023 samples.

5.6.2. Analysis of hair from different body parts

In the analysis of hair from different body parts no statistically significant differences were found in the concentrations of all three hormones (cortisol, testosterone, and progesterone) between the withers and loin areas in the 2022 and 2023 samples of the Caspian seal. Since hair renewal does not occur simultaneously across all body areas of seals, the absence of differences in hormone concentrations in hair from different body regions may indicate the absence of significant variability in the hormonal status of animals during molting.

Despite the lack of statistically significant differences in the studied samples, some individuals exhibited hormone concentration variations of 5-20 times between hair from different body regions. For instance, in individual PC23-17, the cortisol concentration in hair from the lumbar region was 53.59 ng/g, while in the withers region it was 743.97 ng/g. These disparities complicate the combination of these indicators for further comparative analysis.

5.6.3. Cortisol

Cortisol is a glucocorticoid secreted by the adrenal glands in response to activation of the hypothalamic-pituitary-adrenal pathway when the body is exposed to stressors. The complex action of glucocorticoids on the organism is part of the mechanism of adaptation to stress, making cortisol an indicator of the organism's well-being.

Cortisol concentrations in the blood reflect the hormonal status of the animal during the period of sample collection. However, interpreting the results of cortisol analysis when working with Caspian seal data is challenging. The duration and amplitude of the stress response often correlate with the general health of the animal and the presence of chronic stress [Boonstra et al., 1998; Sapolsky, 1993].

The absence of statistically significant sex and age differences may indicate similar reactions to capture among individuals of different sexes and age categories. The detected interannual differences in the concentration of cortisol in the blood of young males may be related to the presence of chronic stress in individuals in 2023. In the presence of chronic stress, an increase in the amplitude of the stress response may be observed [Sapolsky, 1993]. The presence of eye pathology probably does not lead to an increased amplitude of the response to the capture process, as blood cortisol concentrations in most individuals are close to average values.

In 2023, young female Caspian seals had higher hair cortisol concentrations than adult females, but no such pattern was observed in 2022 (analysis of the 2019 and 2020 samples was difficult due to the small number of adults).

A higher concentration of cortisol in the hair of young animals compared to older animals has been demonstrated in some mammals, including pinnipeds [Keogh et al., 2020], as evidenced by studies on the Baikal seal (unpublished data). These differences may be related to the varying degree of success in molting among animals of different ages.

In the distribution of cortisol in the vibrissae of seals sampled in 2023, an increase in concentration closer to the distal end of the vibrissae is observed. The distal end of the vibrissae likely correlates with the molting period. For young animals, higher concentrations of cortisol in vibrissae were observed compared to adult females (both pregnant and non-pregnant), consistent with the results of hair analysis.

It should be noted that in 2022, the increase in cortisol in distal segments was weaker, which may indicate an increase in the impact of some environmental factor on the Caspian seal during the onset of molting in 2023.

Individuals from the 2023 sample exhibited elevated hair cortisol levels in young females and pregnant individuals compared to previous years. In the spring of 2023, when these animals were molting, there was a prolonged presence of sea ice (ice persisted until March 27). This created favorable conditions for molting, so elevated hair cortisol concentrations are likely an indirect indication of any additional stressors during this season.

5.6.4. Testosterone

Testosterone is a sex hormone of the androgen group that has a wide spectrum of action on the body's organ systems. Its primary function is to develop and maintain the functioning of the male reproductive system. Mammals with seasonal reproduction experience an increase in testosterone concentration during the mating period, returning to baseline levels outside the breeding season, a characteristic that can be observed in both males and females [Naidenko et al., 2022].

The absence of sex differences in blood testosterone concentration in young animals is likely due to the absence of sexual activity in this age group. The increased concentration of testosterone in the blood of adult females compared to young individuals may indicate testosterone variability outside the breeding season, warranting further research.

The process of the end of the mating period preceding the molt is reflected in testosterone concentrations in vibrissae segments, where a decrease in testosterone concentration from the tips of the vibrissae to the base is observed. This pattern is consistent not only for Caspian seals in the 2022 sample but also for Baikal seals and largha seals (unpublished data). The absence of pronounced sex and age differences in testosterone concentration in hair is in line with the results of vibrissae analysis.

5.6.5. Progesterone

Progesterone is a sex hormone whose main function is to preserve and maintain pregnancy.

The concentration of progesterone in blood serum can serve as an indicator of pregnancy in females of many mammalian species, including pinnipeds [Reijnders, 1990; Gardiner et al., 1996; Hall et al., 2020]. In the last few months of pregnancy, the concentration of progesterone in the blood increases, allowing pregnant females to be distinguished from non-pregnant ones during this period. In the Caspian seal, progesterone concentration increases in the last few months of pregnancy, as evidenced by the elevated concentration of sex hormones in the hair of aborted embryos (data from 2022).

The analysis of the 2022 Caspian seal sample revealed ranges of blood progesterone concentrations that can suggest pregnancy: concentrations greater than 200 ng/mL indicate a high probability of a female being pregnant, while concentrations of 100 to 200 ng/mL indicate a possible pregnancy. In the 2023 sample, all females identified by ultrasound diagnosis as "pregnant" (PC23-01, PC23-07, PC23-11, PC23-13) had blood progesterone concentrations above 100 ng/mL, but only one individual had a concentration above 200 ng/mL (PC23-13). Three adult females (PC23-04, PC23-06, PC23-08), one young male (PC23-15), and one young female (PC23-16) identified by ultrasonography as "probably not pregnant" also had blood progesterone concentrations above 100 ng/mL. One "probably not pregnant" female had progesterone concentrations exceeding 200 ng/mL (PC23-04). Considering the results obtained during ultrasonic diagnostics and hormonal studies, it can be concluded that pregnancy is likely in 9 out of 13 adult female Caspian seals (PC23-01, PC23-04, PC23-06, PC23-07, PC23-08, PC23-11, PC23-13).

Based on the results of ultrasonic diagnostics and progesterone concentrations in blood, groups of pregnant and yearling females were identified in samples from previous years. The percentage of ulcers ranged from 0 to 40% in 2022 and was 52.8% in 2023. The absence of ulcers among deceased females, the detection of aborted embryos and traces of aborted pregnancies in live females in 2022, and the high percentage of ulcerated females in both samples of live females may indicate that female ulceration is not mainly due to a low percentage of mated females but rather to a high risk of embryo loss in the later stages of pregnancy.

The molting period of Caspian seals coincides with the time of embryonic diapause in pregnant females, during which the concentration of progesterone has low values compared to later periods [Reijnders, 1990], which may be the reason for the absence of age differences in the concentration of progesterone in hair and the uniform distribution of progesterone along the vibrissae of adult individuals, similar to that of young males.

In 2023, juvenile and adult females exhibited elevated levels of progesterone in their hair compared to 2022. The increased levels of progesterone in the blood of juveniles and yearling females in 2022-2023 compared to previous years may be attributed to environmental stressors during the fall (as progesterone is one of the products in the synthesis of cortisol).

5.6.6. Estimating the percentage of females that are pregnant

No pregnant females were identified in the 2019 and 2020 samples (one adult female in 2019 and 2 adult females in 2020). In 2022, 12 out of 20 live individuals (60%) were determined to be pregnant based on blood progesterone concentrations; upon autopsy of deceased animals, all 12 adult females were found to have a fetus.

In 2023, 7 out of 13 adult females (53.8%) were identified as pregnant, and in 4 females, the presence of a fetus was confirmed by ultrasonic diagnostics. Thus, the percentage of calving in 2022 ranged from 0 to 40%, and in 2023, it was 52.8%.

5.7. Serologic studies

To identify the seropositivity of Caspian seals to various pathogens, the results obtained in 2023 were compared with data collected in 2020 (16 samples, including 3 samples from 2019), 2021 (7 samples), and 2022 (29 samples) (Table 5.7-1).

Table 5.7-1 Summary Data on the Percentage of Seropositivity of Caspian Seals to Different Pathogens During All Years of Research

Pathogen	2019 and 2020 (16 samples)	2021 (7 samples)	2022 (29 samples)	2023 (24 samples)	TOTAL (76 samples)	Average in 4 years
Toxoplasma gondii	0	0	24	13	**13.2**	**9.2**
Herpes simplex virus	0	29	24	0	**11.8**	**13.2**
Trichinella sp.	8	0	0	0	**1.3**	**1.9**
Morbillivirus	25	71	83	67	**64.5**	**61.5**
Mycoplasma sp.	77	14	17	71	**43.4**	**44.8**
Chlamydia sp.	93	71	72	79	**76.3**	**79.0**
Candida sp.	92	71	83	92	**82.9**	**84.5**

Among the examined animals, only 4 animals tested negative. Most of the animals were seropositive to 3 pathogens simultaneously (20 seals), with slightly fewer being seropositive to 2 or 4 pathogens simultaneously (16 seals each). No seals were seropositive to all 7 pathogens simultaneously. The maximum number of positive tests recorded simultaneously was for 6 pathogens (in two seals) (Figure 5.7.1).

Figure 5.7.1 Number of Animals Seropositive to Different Numbers of Pathogens

No seals were seropositive for Trichinella in 2023. Over the course of 4 years of research, only one animal (in 2019) out of 76 analyzed tested positive for Trichinella, representing just over 1% both overall and on average.

No seropositive animals were detected for herpes simplex virus in 2023. However, in previous years of studies, the percentage of seropositivity to this pathogen reached almost 30%. In total, 9 out of 76 animals (11.8% overall, 13.2% on average) tested positive for this pathogen during the 4 years of research.

Seropositive animals were detected for the remaining 5 pathogens tested in 2023.

Seropositivity for Candida was highest in 2023 (92%). Although not as high in previous study years, it never fell below 70% (Figure 5.7.2A). A total of 63 seals out of 76 (82.9%) tested positive for Candida in all study years. On average, 80.2% of seals were seropositive each year.

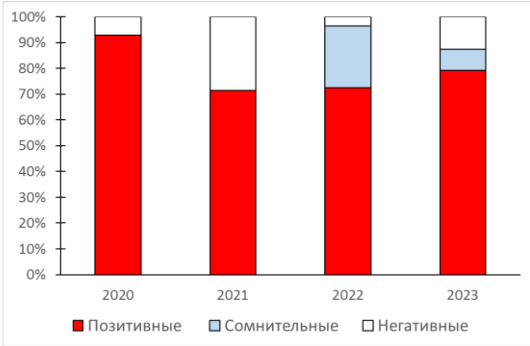
The second most frequent pathogen in 2023 was Chlamydia, affecting 79% of the examined animals. Seropositivity to Chlamydia remained consistently high across all years, never dropping below 71%. Overall, among all examined animals, it amounted to 76.3%, with 79% of seals being seropositive on average per year (Figure 5.7.2B).

The situation with seropositive animals to the carnivore plague virus from the morbillivirus group was particularly interesting (Figure 5.7.2C). Over the four years, it averaged 64%, with an average of 61.5% of seropositive seals detected each year. In 2023, it remained relatively high at 67%, slightly lower than in 2022 (83%) but significantly higher than in 2020 (25%). Overall, the proportion of animals seropositive for the rinderpest virus showed a clear trend: a sharp increase in 2021 compared to 2020, peaking the following year, and decreasing in 2023.

The proportion of mycoplasma-seropositive animals averaged 45% over the four years (Figure 5.7.2D). In 2023, the proportion of seropositive animals increased to 71%, significantly higher than in the previous two years and on par with the level observed in 2020.

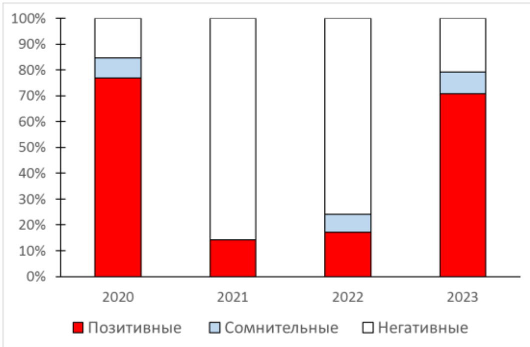
The proportion of animals seropositive for toxoplasma was low at only 13% (average of 9.2% per year) (Figure 5.7.2E). However, it was not observed at all in the first two years, with the highest values recorded in 2022 and the same 13% in 2023.

A) to candida

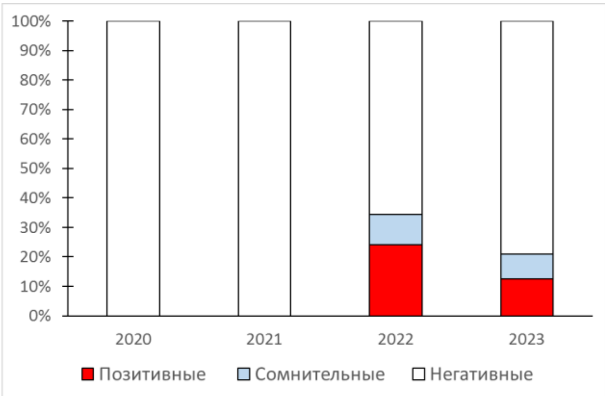


B) to chlamydia

C) to the rinderpest virus



D) to mycoplasma



E) to toxoplasma

Figure 5.7.2 Percentage of Seropositive Animals

Toxoplasma (*Toxoplasma gondii*) is one of the most widespread parasites globally, capable of infecting virtually all warm-blooded organisms. Toxoplasma is also prevalent among marine mammals, including pinnipeds. Previous assumptions suggested that seropositivity to toxoplasma among Caspian seals exceeds 80% [Namroodi et al., 2018]. However, recent findings indicate that while all adult animals have antibodies to toxoplasma, only 60% of individuals under one year of age were seropositive to this pathogen. Toxoplasmosis can lead to immunosuppression in hosts and may even result in the death of animals [Greene, 2006]. Seropositivity to toxoplasma rarely exceeded 20%, averaging 13%. Kazakhstani scientists, based on a 10-year study, reported that antibodies to toxoplasma were detected in only 2.6% of animals [Kydyrmanov et al., 2023].

Morbilliviruses have been repeatedly considered the cause of death in various pinniped species, including the Caspian seal [Pollack et al., 2001; Jo et al., 2019]. Diagnosing seropositivity to different forms (carnivore plague virus, seal plague virus, and cetacean morbillivirus) is extremely difficult because the viruses share significant similarities, leading to cross-reactivity of antibodies produced by animals to all three pathogens. The kits used to detect antibodies to carnivore plague virus also showed cross-reactivity to seal plague virus. The virus or viruses appear to circulate in the population, sometimes resulting in mass mortality events. In November 2022, antibodies to morbillivirus were detected in 83% of Caspian seals.

The high percentage of seropositive animals in 2021 and even higher in the fall of 2022 indicates the development of an epizootic in the population during this period. However, by 2023, a decrease in the proportion of seropositive animals was observed. Interestingly, in earlier periods, the seropositivity of Caspian seals to CDV was significantly lower (8% from 2007 to 2017) [Kydyrmanov et al., 2023].

In the winter of 2022-2023, there were cases of mass seal mortality, which, according to the analyses conducted, were not caused by viral agents. In this scenario, it becomes intriguing why a virus consistently found in Caspian seals only leads to mass mortality in rare instances. Perhaps the reason also lies in pathogen mutations, as demonstrated, for example, in the case of the African lion [Nikolin et al., 2017]. Another plausible explanation could be the presence of antibodies in animals following an epizootic, although unlike the one described in 2019 [Jo et al., 2019], such cases have not been documented in publicly available sources. Long-term studies and regular sampling, including during periods of animal mortality, will provide a clearer understanding of the situation.

Research on seropositivity of Caspian seals to the other pathogens listed began in 2020. The presence of Trichinella in various pinnipeds, including ringed seals, lahtak, and walruses, has been confirmed by several authors [Roth, Madsen 1953]. Since Trichinella circulation in nature is linked to the consumption of meat from warm-blooded animals, it is assumed that in populations where walruses more frequently prey on other pinnipeds, the level of trichinellosis should be higher. However, the proportion of seropositive animals was low, ranging from 0.2% to 13.5% [Fay, 1960]. It is likely that all pinnipeds, including walruses, play a crucial role in transporting Trichinella in ecosystems, potentially infecting humans when consumed. In 2022-2023, no Caspian seals seropositive for Trichinella were detected.

Detection of antibodies to Candida sp. in almost all animals indicates its widespread distribution in the region. Candida, along with mycoplasma and chlamydia (the most common pathogens in Caspian seals), likely contribute to weakening the immune system and increasing the risk of infection with other pathogens rather than leading to more severe consequences for the animals. Mycoplasma has been frequently observed in gray and harbor seals, including its presence in the oral cavity of animals, which consequently affects areas where animals bite each other on fins and skin [Ayling et al., 2011]. Although the presence of Candida in Caspian seals has not been previously described, it has been observed in other marine mammals, particularly beluga whales.

Monitoring seropositivity of Caspian seals to these pathogens and several others is necessary for multiple years to assess the overall well-being of the population, including factors affecting its seasonal changes.

5.8. Immunity Studies

The study revealed that the activity level of the complement system in Caspian seals, as determined in the bacterial killing test, was significantly higher than in most terrestrial mammals. The average index was 3, corresponding to titer 8, whereas in most terrestrial predators, it corresponded to 2 or 4. Additionally, the concentration of immunoglobulin G in Caspian seals was found to be twice as low as in harbor seals.

No significant age differences were found in the majority of immune system activity indicators, suggesting that the immune system is fully developed in animals by the age of one year. However, the level of lysozyme in the blood of semi-adult and sexually mature animals was notably higher than in young animals. One possible explanation for this disparity could be pregnancy among animals in the first two age groups. Semi-adult animals aged 4-5 years showed a very high level of testosterone, indicating probable pregnancy. It appears that during pregnancy, lysozyme, an enzyme that effectively targets the walls of gram-positive bacteria, becomes crucial for successful reproduction in seals.

Thus, the specificity of the Caspian seal immune system is characterized by a relatively low level of immunoglobulins (across all classes and IgG), coupled with high antibacterial activity in sera (both in bacterial killing tests and lysozyme concentrations). These innate immune features likely enable the seals to successfully resist various bacterial pathogens.

6. CONCLUSION

Monitoring the status of marine mammal populations is one of the most important areas of ecological research, serving as an integral component of wildlife conservation strategies and the control of potential threats to human and animal health. Marine mammals in many waters are used as indicator species for medium- and long-term observations of changes in the external environment (Aguilar, Borrell, 1994).

The Caspian seal, Pusa caspica (Gmelin, 1788), is the only marine mammal inhabiting the Caspian Sea. It is endemic and plays a unique role in its ecosystem, serving as the closing link in the trophic chain of the Caspian aquatic ecosystem. Inhabiting the entire sea area, it can therefore be considered an indicator species of its condition.

The territorial waters of Kazakhstan and Russia in the Caspian Sea, especially the northern part, are the most important areas for the Caspian seal. From fall to early spring, the main part of the population migrates across the sea, concentrating in the Northern Caspian for one of the most important stages of the life cycle - breeding.

As a result of virological analysis conducted in 2023 on samples from Caspian seals, no nucleic acids of pathogens such as influenza (influenza A and B viruses), coronavirus, paramyxovirus (including carnivore morbillivirus), hepivirus (hepatitis E), poxvirus, lyssavirus, or retrovirus infections were detected. However, PCR products corresponding to the expected sizes of adenovirus and herpesvirus gene fragments were found in nasal wash samples collected in 2023 from animals exhibiting signs of respiratory infection. These findings suggest the presence of mixed respiratory infections among the studied group of animals, caused by adenoviruses and herpesviruses of seal serotype 2. Given the dominant role of viruses in infectious pathology, further research is warranted.

In the virome of Caspian seals, we have identified two large groups consisting of different families of viruses. The first group comprises the families Circoviridae and Parvoviridae, which are associated with the aquatic ecosystem. The primary hosts of these viruses are various organisms in the marine environment of the Caspian Sea, such as invertebrates in the case of circoviruses, or insects and crustaceans in the case of densoviruses of the family Parvoviridae. Notably, this group accounts for 72% of the total Caspian seal virome, suggesting a possible dietary origin. The second group encompasses mammalian viruses, including Herpesviridae, Papillomaviridae, Caliciviridae, Anelloviridae, Adenoviridae, Orthomyxoviridae, and Paramyxoviridae. Viruses in this group have the potential to cause various pathologies in mammals or to be asymptomatic.

Taxa representing 22 types of bacteria were found in the microbiome of Caspian seals, with five types predominating in the dataset: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Fusobacteria. Similarities in the microbiome profiles of respiratory, alimentary, and urogenital tract flushes of animals collected from 2020 to 2023 were established. Variation in the percentages of bacterial families was noted depending on the type of sample from Caspian seals. This study indicates the need for continuous monitoring of the microbiome of Caspian seals to detect the introduction of clinically significant bacterial pathogens into their population. Overall, the results of this study provide a solid foundation for future research and contribute to the understanding of host-microbe interactions in the Caspian seal population.

The obtained serological data complement the results of PCR screening for the presence of influenza A virus in the samples of seals that were collected in the Kazakh and Russian parts of the sea at the end of 2022, indirectly indicating the involvement of the pathogen in an outbreak of epizootic influenza infection among seals with high mortality. Antibodies to CDV were not detected in the sera of seals collected in 2020 but were detected in up to 28% of the samples collected in 2022. Additionally, one serum obtained in 2019 and two samples in 2023 were positive for antibodies to carnivore plague virus. These findings suggest a recent outbreak of the infections in these animal populations.

According to the results of studies on 22 samples of seal feces using the Darling and Scherbovich methods, no helminth eggs, including those of the helminth Anisakis chupakovi, were found. The absence of helminth eggs in the feces of seals may indicate either a small sample size of the material under study or the absence of infested seals during the study period.

The preparation of libraries from biological samples of Caspian seals for the characterization of the microbiome of the respiratory and gastrointestinal tracts through sequencing of the 16S ribosomal RNA gene of the microflora was carried out.

Microbiological studies in 2023 revealed that the microbiome of the Caspian seal mucous membranes includes six dominants that are found only in pinnipeds. The rectal and urogenital microbiome lacks eurybiont microorganisms and is composed of 90% bacteria that exist in other pinnipeds. The nasal microbiome is highly conserved and stable compared to the nasal microbiota of many other marine mammals, and it is typically devoid of Streptococcus species.

Enterobacteriaceae are almost completely absent in the rectal and urogenital microbiomes. Lactobacilli are also almost entirely absent in the urogenital microbiome. Representatives of Pasteurellaceae and Campylobacteriaceae are part of the normal microbiota of the Caspian seal and cannot be considered pathogens.

The microbiome of Caspian seal mucous membranes is capable of metabolizing carbohydrates, lipids, and proteins. The communities in the urogenital and rectal mucosa are more prone to rapid anaerobic digestion of carbohydrates. On the other hand, the nasal microbiome tends to engage in aerobic metabolism of carbohydrates and fatty acids via the citrate and methylcitrate pathway.

The gut microbiome of the Caspian seal lacks the capability to ferment food polymers such as cellulose, pectins, and chitin, but it can hydrolyze peptides and aminoglycans. Therefore, seals are unable to digest the structural polysaccharides of algae and the chitin shells of crustaceans.

The low antibiotic resistance, regardless of mucosal localization, indicates that animals are not affected by agricultural plums, which are usually rich in vancomycin.

The developed defense system against aromatic-type xenobiotics for all mucous membranes indirectly indicates the presence of anthropogenic aromatic-type pollutants in the water, to which the nasal microbiome primarily responds.

Toxicological studies conducted in 2023 aimed to detect mercury in the blood, hair, and vibrissae of Caspian seals.

Mercury concentrations in hair ranged from 258 µg/kg to 10,285 µg/kg, in vibrissae from 954 µg/kg to 12,957 µg/kg, and in blood from 29 to 350 µg/liter. The levels of mercury accumulation obtained in the Caspian seal organism fall within the range of values typical for other pinnipeds in both Arctic and temperate latitudes.

Mercury concentrations in Caspian seal biomaterials remain consistent throughout the study period, indicating stable pollutant levels in the Caspian Sea and the absence of significant inputs of mercury.

The mercury content in all studied biomaterials does not depend on the sex of seals, indicating a homogeneous diet among males and females. Mercury concentration in vibrissae is higher than in Caspian seal fur, possibly due to a longer period of mercury accumulation in vibrissae between their changes. Mercury concentrations in all biomaterials are statistically significantly positively correlated with each other.

Mercury concentrations in hair and vibrissae gradually increase with age, but it is unclear whether this is due to accumulation in the body or to the diet of seals of different ages. Blood shows negative correlations with age and body size.

Throughout the entire study period, mercury concentrations above the values at which neurotoxic effects can occur were observed in 11% of the total number of seals tested in coat analysis and 5% in blood analysis. For the majority of the studied individuals, mercury concentrations do not pose a health risk and are unlikely to cause damage to the population.

The results of studies on the hormonal and immune status of Caspian seals in 2023 significantly complement earlier research and demonstrate the possibility of determining key indicators of the animals' physiological state.

However, even the results of studies conducted over three years show significant inter-annual differences, such as variations in seropositivity of animals to certain pathogens, which allows for the detection of the course of epizootics (e.g., morbillivirus in 2022). This underscores the need for regular monitoring studies of Caspian seals using minimally invasive methods in different parts of their range.

Animal tracking: The data obtained, in addition to those from works in 2019, 2020, and 2022, enhance the understanding of Caspian seal migrations. Several patterns are observed in animal movements, but the sampling required to comprehend all variants of Caspian seal migratory behavior is insufficient.

The primary area of the seals' habitat during the autumn-winter months is in the northern part of the Caspian Sea, although individuals venture into the waters of the middle Caspian Sea and even reach its southern regions.

The distribution of animals in February-March correlates directly with the distribution of ice cover. In the winter of 2019-2020, a year with low ice cover, animals were mainly distributed in the eastern part of the North Caspian, close to the coastline. In the wetter winters of 2020-2021 and 2022-2023, ice covered most of the North Caspian Sea, and seals were located at its edge.

For animals tagged in 2023, as well as those tagged in 2020 and 2022, there is a more active use of the middle part of the Caspian Sea compared to the 2019 tagging. Additionally, in these years, active use of the eastern coast of the Caspian Sea is noted, whereas for the 2019 tagging, seals more frequently used the water area near the western coast of the Caspian Sea for movements.

The conducted studies confirm the presence of a "migration corridor" in the eastern part of the Caspian Sea off the coast of Kazakhstan, as noted earlier in the literature (Dmitrieva et al., 2016).

7. RECOMMENDATIONS

Despite the increased number of studies on the Caspian seal in the Kazakhstan sea area in recent years, many aspects of its biology and ecology remain unstudied or poorly studied.

All seal distribution studies in recent years have been conducted locally. The existing modern data on the abundance and distribution of seals during winter and early spring periods in the KSCM (pupping and molting periods) provide valuable scientific information, but there is no comprehensive understanding of the modern distribution of seals during the summer in the Northern Caspian Sea.

Recommended approaches for exploring and co-processing the resulting data

– Distribution, abundance, and migration routes:
  - *Aerial surveys throughout the North Caspian Sea (minimum of five consecutive years, covering all four seasons);*
  - *Survey of seal haulouts using remote sensing methods (including drone surveys, remote sensing data, and photo traps);*
  - *Continuation of satellite tagging to study seal migration routes in different seasons and years. Tagging should be carried out after the complete molt of animals in spring and/or fall;*
  - *Shipboard observations on the distribution and concentration of seals in the Caspian Sea water area during different seasons of the year. Observations should cover routes of ships, areas of small-sized fleet operation, offshore islands, and sand islands;*
  - *Development of a unified GIS system to collect data on resting, feeding, and denning areas.*

– Population structure:
  - *Genetic studies to either prove or disprove the hypothesis about the existence of several subpopulations of the Caspian seal.*
  - *Demographic structure analysis of the population, including the creation of tables showing the dependence of linear sizes on age.*

– Welfare of the Caspian seal population:
  - *Priority tasks for virological research, including the detection of clinically significant viral pathogens, analysis of seal blood serum for seropositivity to plague virus, toxoplasma, influenza A and B virus, trichinella, mycoplasma, chlamydia, and pasteurella.*
  - *Parasitological studies, including complete helminthological autopsy according to K.I. Skryabin (for deceased seals), animal fecal examination, and DNA metabarcoding of fecal samples and rectal flushes.*
  - *Microbiological studies, focusing on the microbiome of the respiratory and gastrointestinal tracts of seals through sequencing the 16S ribosomal RNA of microflora.*
  - *Toxicological studies, involving the analysis of subcutaneous fat, liver, kidney, etc., for components such as petroleum products, COPCs, TM, phenols, and biogenes.*
  - *Assessment of Caspian seal nutrition, examining seal nutrition through feces analysis and fatty acid analysis in biopsy samples collected via minimally invasive methods.*
  - *Assessment of hormonal status, including the development of a method to assess the level of animal stress.*
  - *Collection of materials from live and deceased seals, involving the development of a common protocol for measurements, post-mortem examinations, and biomaterial collection, accounting for both live and deceased animals.*

– Caspian seal habitat condition (habitat quality assessment):
  - *Ice conditions and their dynamics, including remote sensing of the Earth using radar satellite imagery, interpretation, and analysis of space images.*

- *Identification of seal haulouts during the ice-free period, tracking their dynamics, creation of a constantly updated map of such sites, entry of this data into the GIS database being created, and planning for the future. This also involves modeling habitat suitability for seals during the ice-free period.*
- *Monitoring of anthropogenic load, which includes monitoring of oil production and transportation accidents, assessment of vessel traffic, and monitoring of pollution in the main feeding areas and Caspian seal pup areas.*
- *Control of water quality and bottom sediments in the North Caspian Sea in accordance with the List of determinable components and environmental indicators, as specified in the "Rules of organization and conduct of baseline environmental studies during oil operations in the KSCM" [Order of the Minister of Energy of the Republic of Kazakhstan from 07.09.2015 № 559].*

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APPENDIX A SCOPE OF WORK

SURVEY OF CASPIAN SEALS IN THE WATER AREA OF THE NORTH CASPIAN SEA, AUTUMN

1. INTRODUCTION

To receive reliable data on the abundance and location of Caspian seal populations in the water area of the North Caspian Sea and also to create a database pertaining to the preservation of the seal population, in 2022 the Company Environmental Protection Department committed to:

– determine the current status of the population and its trends in the North Caspian Sea;
– prepare reports with cartographic and photographic materials on work performed;
– prepare academic review publications based on work results.

2 SCOPE OF WORK – GENERAL PROVISIONS

This document sets out the scope of work for the period between 3 February and 29 December 2023, including data processing and preparation of report.

The CONTRACTOR should perform all multispectral aerial surveys of seal herds and surveys of the current status of the Caspian seal population during the 2023 winter and spring periods.

The CONTRACTOR may engage subcontractors to perform survey work with respect to this scope of work, and whose services will be reimbursed according to the terms and conditions of this Contract UI176768.

Survey objectives: Assess the abundance, distribution and welfare of the Caspian seal population in the water area of the North Caspian Sea in 2023.

Survey goals:

– create a multispectral aerial survey of the abundance of the population breeding stock and offspring using infra-red, video and photographic tools from an aircraft in accordance with the international work programme to “assess the abundance, distribution and natural reproduction of the Caspian seal in the Kazakhstan and Russian water areas of the North Caspian Sea in 2020-2024, as approved by the authorised state bodies of Kazakhstan and Russia;
– determine the nature and density of the distribution of animal ice herding areas;
– determine the correlation between the number of females and young;
– provide aerial surveys of seal rookeries and moulting areas in spring;
– capture seals, perform morphometric studies, collect biological material for toxicological, physiological (serological and hormonal), virusological and microscope survey according to the Programme to “Survey the Caspian seal in the water area of the North Caspian Sea in 2019-2023”, approved by the Company on 3 July 2019;
– to study the routes of the Caspian seal movement using satellite telemetry by installing up to 10 satellite radio beacons on captured animals in the waters of the North Caspian Sea in autumn period;
– record the abundance of seals on vessel routes, at rookery sites, and where field work is performed during the autumn;
– carry out preliminary processing on aerial survey materials, verify materials, conFigure them and prepare them for an assessment of the abundance of Caspian seals;
– map the locations of seal concentrations;
– develop proposals for preserving seal populations;
– prepare analytical reports.

Surveys are performed in two stages:

- winter aerial surveys of the abundance of seals on ice rookeries in February;
- spring aerial surveys of rookeries and seal moulting areas; installation of satellite radio beacons on captured seals to collect biological samples in November.

The two following contractor instructions are issued within the framework of this scope of work:

an aerial survey of the abundance of seals in winter and spring;

comprehensive seal surveys.

3. SERVICE REQUIREMENTS

3.1 General Requirements

The processing algorithm for multispectral survey materials with respect to Caspian seal breeding grounds incorporates the following stages.

To process materials, the Contractor should, in separate stages:

- decipher the multispectral survey materials (photographic, infrared survey and navigation images) and compile seal estimate summary tables;
- calculate the density of the seal population using instrument data;
- extrapolate seal density to the water area under consideration and make an estimate of seal abundance.

The Contractor should initially decipher all materials received, which include photographic and video images etc. In this respect, television images are processed together with photographic images. Infrared imaging is used to detect seals resting on the ice, while the corresponding photographic images are used to identify seals. Materials should be deciphered using three independent meters, which allows the user to identify calculation errors. Deciphered materials can be used to generate seal data summary sheets in chronological order, in conjunction with survey navigation parameters. The time of day is the same in all tables; any delays and discrepancies are adjusted. Integrated and generally accepted formats are used for different values, such as dates, time, coordinates and others.

The number of seals on each route is then calculated and totalled, after which the total area covered is determined. The density of seal herding (young and adult seals) is then determined based on instrumental survey data.

Seal (pups and adults) density data is subsequently extrapolated to the water area being surveyed to calculate the abundance of seals.

Seal abundance should be calculated using traditional algorithms for extrapolating seal density. Double-sided lower and upper confidence intervals that correspond to the confidence coefficient given (0.95) are calculated together with the weighted average estimate of seal abundance.

Statistical and other errors should be assessed and calculated at each data processing stage. The methods and corresponding formulas for calculating errors are determined depending on the models chosen, assumptions made and restrictions, data filtration and sample compilation methods, as well as area segmentation methods used in extrapolation and other conditions.

The Contractor should transfer all primary (original) materials received from photographic, video and infrared surveys to the Company electronically.

3.2 Requirements for the quality of services

Transect photographic images, as well as video and infrared materials should be analysed by at least three specialists with experience in analysing photographs, video and infrared data from aerial pinniped surveys.

Individual specialist errors in assessments of the abundance of seal pups and adults on metering transects should not exceed 5%.

The results of animal count estimates should be pegged to seal imaging route coordinates.

The statistical analysis of findings from an analysis of photographic, video and infrared surveys should be carried out using at least two special statistical analysis methods (area method and Kinsley et al).

4. REPORTING

The Contractor should provide the Company with independent work Field Reports electronically within 10 working days of the conclusion of winter and spring field work.

Based on the results of processing the received materials on multispectral surveying and studying the current state of the Caspian seal population, the CONTRACTOR should prepare a Detailed final report "Caspian seal records in the North Caspian Sea in winter and spring 2023", which consists of two main sections:

1. Assessment of the abundance and birth rate of the Caspian seal in the water area of the North Caspian Sea in 2023.
2. Study of the current conditions of the Caspian seal population as a species indicator of the state of the Caspian Sea ecosystem.

The structure of the Detailed Final Report should be agreed by the Company.

A preliminary version of the Detailed Final Report should be provided in English and Russian by 29 December 2023.

The final version of the Detailed Report, after approval by the Company, must be submitted both in digital format on electronic media and in hardcover in five copies in Russian and one in English

Reviewed academic publications should be issued on the basis of work findings. At the same time, the Company's participation in the work in question should be referred to in each publication.

APPENDIX B LIST OF BIOLOGICAL SAMPLES

Table B.1 List of Biological Samples Taken by Kazakhstan Microbiology and Virusology Scientific and Production Centre Specialists from Live Seals. November, 2023

Animal number	Type of material / quantity of bioprobes					
	Washing:					Blood serum
	conjunctival	nasal	oral	urogenital	rectal	
PC.PR-11/23-01(A*)	1	1	1	1	1	1
PC.PR-11/23-02(A)	1	1	1	1	1	1
PC.PR-11/23-03(A)	1	1	1	1	1	1
PC.PR-11/23-04(A)	1	1	1	1	1	1
PC.PR-11/23-05(A)	1	1	1	1	1	1
PC.PR-11/23-06(A)	1	1	1	1	1	1
PC.PR-11/23-07(A)	1	1	1	1	1	1
PC.PR-11/23-08(A)	1	1	1	1	1	1
PC.PR-11/23-09(A)	1	1	1	1	1	1
PC.PR-11/23-10(A)	1	1	1	1	1	1
PC.PR-11/23-11(A)	1	1	1	1	1	1
PC.PR-11/23-12(A)	1	1	1	1	1	1
PC.PR-11/23-13(A)	1	1	1	1	1	1
PC.PR-11/23-14(A)	1	1	1	1	1	1
PC.PR-11/23-15(A)	1	1	1	1	1	1
PC.PR-11/23-16(A)	1	1	1	1	1	1
PC.PR-11/23-17(A)	1	1	1	1	1	1
PC.PR-11/23-18(A)	1	1	1	1	1	1
PC.PR-11/23-19(A)	1	1	1	1	1	1
PC.PR-11/23-20(A)	1	1	1	1	1	1
Total:	**20**	**20**	**20**	**20**	**20**	**20**
Grand total of samples:	**120**					

Note: *(A)* – Alive .*

Table B.2 List of Biological Samples Taken by Kazakhstan Microbiology and Virusology Scientific and Production Centre Specialists from Dead Seals. November, 2023.

Number	Gender	Type of material / quantity of biological samples									
		brain	cerebellum	lungs	heart	liver	spleen	kidney	lymph nodes	placenta	intestinal tract
PC.KU-11/23-21(D*)	female	1	-	1	1	1	1	-	1	-	-
PC.KU-11/23-22(D)	male	-	1	1	1	1	1	1	1	-	-
PC.KU-11/23-23(D)	male	-	-	1	-	1	1	-	1	-	1
PC.KU-11/23-24(D)	female	1	1	1	1	-	-	-	-	1	1
Grand total of samples:	**24**										

Note: *(D)* – Dead*

Table B.3 List of Biological Samples Collected by Institute of Ecology and Evolution of the Russian Academy of Sciences Specialists from Live Caspian Seals in November, 2023

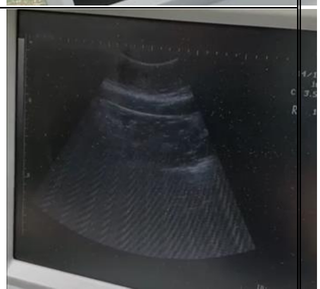
Animal number	Nasal swab, units	Rectal swabs, units	Vaginal swabs, units	Hair for hormonal study, units	Hair for toxicological study, units	Vibrissae for hormonal and toxicological study, units	Whole blood, for heavy metals study, ml	Whole blood, for toxicological study, ml	Blood plasma, number of test tubes	Blood swabs, units	Blood corpuscles for genetic study, number of test tubes
PC23-01	3	3	3	2	1	2	1	9	3	2	2
PC23-02	3	3	3	2	1	2	1	13	3	2	2
PC23-03	3	3	3	2	1	2	1	11	3	2	2
PC23-04	3	3	3	2	1	2	1	17	3	2	2
PC23-05	3	3	3	2	1	2	1	32	3	2	2
PC23-06	3	3	3	2	1	2	1	16	3	2	2
PC23-07	3	3	3	2	1	2	1	12	3	2	2
PC23-08	3	3	3	2	1	2	1	11	3	2	2
PC23-09	3	3	3	2	1	2	1	20	3	2	2
PC23-10	3	3	3	2	1	2	1	14	3	2	2
PC23-11	3	3	3	2	1	2	1	19	3	2	2
PC23-12	3	3	3	2	1	2	1	16	3	2	2
PC23-13	3	3	3	2	1	2	1	15	3	2	2
PC23-14	3	3	3	2	1	2	1	2	3	2	2
PC23-15	3	3	0	2	1	2	1	23	3	2	2
PC23-16	3	3	3	2	1	2	1	1	3	2	2
PC23-17	3	3	3	2	1	2	1	5	3	2	2
PC23-18	3	3	0	2	1	2	1	6	3	2	2
PC23-19	0	0	0	2	1	2	1	8	3	2	2
PC23-20	0	0	0	2	1	2	1	11	3	2	2
Total:	**54**	**54**	**48**	**40**	**20**	**40**	**20**	**20 samples**	**60**	**40**	**40**
Grand total of samples:	**436**										

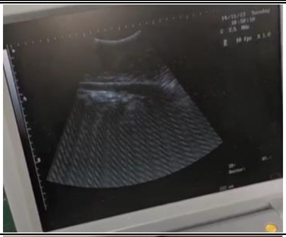
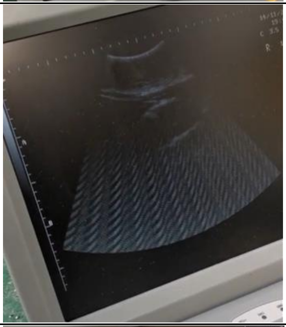
APPENDIX C TABULATED DATA ON STUDY FINDINGS

Table C.1 Meteorological conditions during the work period, 2023

Date	Time	Wind speed (m/s)		Wind direction	Air temperature, ^{0}C	Atmospheric pressure (Mmhg)	Cloud cover (octant) / precipitation	Comments
		mean	gusts (max)					
06.11.2023	6:00	9	12	SSE	4	763	4	Mobilisation
	12:00	8	14	SSE	8	763	4	
	18:00	9	12	SSE	16	764	2	
	24:00	11	13	SSE	14	764	2	
07.11.2023	6:00	6	8	WSW	4	764	9	Mobilization. Sea sortie
	12:00	9	12	WSW	9	764	9	
	18:00	7	10	WSW	14	764	7	
	24:00	7	10	WSW	12	764	7	
08.11.2023	6:00	10	15	WSW	7	763	3	Expected improvements to weather conditions. 1m wave height
	12:00	10	13	WSW	12	762	2	
	18:00	12	15	SW	14	763	1	
	24:00	10	12	WSW	8	763	1	
09.11.2023	6:00	7	10	ESE	4	764	3	Expected improvements to weather conditions. Up to 1.2 m wave height
	12:00	12	14	ESE	9	765	1	
	18:00	14	18	SES	13	765	1	
	24:00	12	16	SES	8	765	1	
10.11.2023	6:00	7	11	WSW	2	766	1	Expected improvements to weather conditions
	12:00	11	15	WSW	12	766	3	
	18:00	11	14	SW	12	766	8	
	24:00	12	16	WSW	8	767	9	
11.11.2023	6:00	3	5	N	2	770	1	Sortie on two working boats to the marine sand island region. Registration of a seals from a boat
	12:00	3	4	NE	7	770	2	
	18:00	5	5	N	8	772	1	
	24:00	3	4	NE	6	773	2	
12.11.2023	6:00	8	10	ESE	5	774	2	Expected improvements to weather conditions
	12:00	10	15	ESE	10	774	2	
	18:00	12	16	ESE	9	774	1	
	24:00	7	9	ESE	6	774	1	
13.11.2023	6:00	12	15	SE	6	774	4	Sortie on two working boats to the marine sand island region. Tagging and sampling.
	12:00	7	10	SE	10	775	4	
	18:00	2	4	ESE	10	775	2	
	24:00	7	10	ESE	2	775	2	
14.11.2023	6:00	10	14	SE	6	775	2	Sortie on two working boats to the marine sand island region. Tagging and sampling.
	12:00	8	10	SE	11	774	2	
	18:00	1	2	SE	10	774	4	
	24:00	6	11	E	7	774	5	
15.11.2023	6:00	8	12	NNW	5	775	4	Seal tissue sampling.
	12:00	5	8	NNW	10	775	3	
	18:00	5	7	W	12	775	2	
	24:00	7	10	W	7	775	2	
16.11.2023	6:00	6	10	SE	6	775	6	Seals registration along the vessel movement route
	12:00	7	12	SSE	13	776	7	
	18:00	3	10	SE	15	776	9	
	24:00	5	9	SE	8	775	9	
17.11.2023	6:00	6	9	SES	7	775	7	Demobilization
	12:00	6	11	NW	10	776	7	
	18:00	7	11	W	11	776	5	
	24:00	10	15	NW	8	775	5	

Table C.2 Ultrasonic examination of seal female

Number of animal	Age, years	Age category	Pregnancy based on ultrasound results	Ultrasonic examination photo
PC23-01	4	adult	pregnant	
PC23-02	4	adult	probably, not pregnant	
PC23-03	5	adult	probably, not pregnant	
PC23-04	8	adult	probably, not pregnant	
PC23-05	6	adult	probably, not pregnant	
PC23-06	4	adult	probably, not pregnant	

Number of animal	Age, years	Age category	Pregnancy based on ultrasound results	Ultrasonic examination photo
PC23-07	5	adult	pregnant	
PC23-08	5	adult	probably, not pregnant	
PC23-09	7	adult	probably, not pregnant	
PC23-10	5	adult	probably, not pregnant	
PC23-11	6	adult	pregnant	

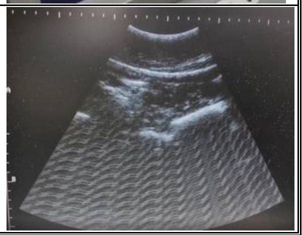
Number of animal	Age, years	Age category	Pregnancy based on ultrasound results	Ultrasonic examination photo
PC23-12	5	adult	probably, not pregnant	
PC23-13	6	adult	pregnant	
PC23-14	2	young	there is a high probability that there is no pregnancy	

Table C.3 Findings from Morphometric Studies on Live Seals, 2023.

Animal number	Capture location coordinates	Study date	Study location	Gender	Age, years	Weight, kg	Zoological body length, cm	Chest cavity circumference, cm	Tail length, cm	Comments
PC23-01	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	4	71	116	119	9	Awake, no injuries
PC23-02	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	4	73,1	120	130	9	Awake, no injuries
PC23-03	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	5	66,8	120	115	8	Awake, ticks were found on the flippers
PC23-04	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	8	74,7	120	125	7	Awake, scar on the left side and scars, wounds on the right pow; irritation at the anus
PC23-05	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	6	73,3	124	125	11	Awake, no injuries
PC23-06	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	4	78,5	127	115	10	Awake, a scar and a wound near the mouth
PC23-07	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	5	82	122	132	8	Awake, no injuries
PC23-08	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	5	79,1	114	128	9	Awake, small scars on the hind limbs, missing several claws on the hind limb, greenish-yellow discharge from the eyes
PC23-09	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	7	56,7	117	117	10	Awake, the back and paws are in large and small scars, there are small bald areas of the body, there is no claw on the left front paw
PC23-10	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	5	65,9	116	117	7	Awake, small scars on the head
PC23-11	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	6	68	109	118	7	Awake, several small scars and a wound on the back, the claw on the front paw is broken
PC23-12	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	5	72,5	108	115	10	Awake, the claw wound on the right front paw
PC23-13	45.95093° N; 52.264° E	13.11.2023	RK, sand island near the NCMC	female	6	80,4	133	128	7	Awake, no injuries
PC23-14	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	female	2	24,2	88	85	8	Awake, no injuries
PC23-15	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	male	2	21,3	100	83	8	Awake, no injuries
PC23-16	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	female	2	20,2	91	81	7	Awake, scars on the back flipper
PC23-17	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	female	1	25,5	94	87	9	Awake, irritation of the mucous membrane of the eyes
PC23-18	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	male	3	30	96	96	7	Awake, there are no several claws on both front paws

Animal number	Capture location coordinates	Study date	Study location	Gender	Age, years	Weight, kg	Zoological body length, cm	Chest cavity circumference, cm	Tail length, cm	Comments
PC23-19	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	female	2	20	85	78	9	Awake, no injuries
PC23-20	45.95093° N; 52.264° E	14.11.2023	RK, sand island near the NCMC	female	3	33,6	97	99	7	Awake, no injuries

Table P.4 Data on tagged Caspian seals in November, 2023

Animal number	Capture / release location	Transmitter installation date	Gender	Age, years	Transmitter installation time (glue setting), hours (UTC + 5)	Seal release time, hours (UTC + 5)	Transmitter fixture location	Transmitter number
PC23-01	RK, sand island near the NCMC	13.11.2023	female	4	10:30	15:00	between the shoulders	253210
PC23-02	RK, sand island near the NCMC	13.11.2023	female	4	11:42	15:00	between the shoulders	253211
PC23-03	RK, sand island near the NCMC	13.11.2023	female	5	12:35	15:01	between the shoulders	253212
PC23-04	RK, sand island near the NCMC	13.11.2023	female	8	13:08	15:01	between the shoulders	253213
PC23-05	RK, sand island near the NCMC	13.11.2023	female	6	14:09	15:02	between the shoulders	253214
PC23-06	RK, sand island near the NCMC	13.11.2023	female	4	15:47	22:14	between the shoulders	253215
PC23-07	RK, sand island near the NCMC	13.11.2023	female	5	16:21	22:16	between the shoulders	253216
PC23-08	RK, sand island near the NCMC	13.11.2023	female	5	17:11	22:09	between the shoulders	253217
PC23-09	RK, sand island near the NCMC	13.11.2023	female	7	18:09	22:13	between the shoulders	253218
PC23-10	RK, sand island near the NCMC	13.11.2023	female	5	18:39	22:07	between the shoulders	253219
PC23-11	RK, sand island near the NCMC	13.11.2023	female	6	19:10	22:05	between the shoulders	253220
PC23-12	RK, sand island near the NCMC	13.11.2023	female	5	19:30	22:03	between the shoulders	253221
PC23-13	RK, sand island near the NCMC	13.11.2023	female	6	20:00	22:10	between the shoulders	253222

APPENDIX D VIROME DATA ANALYSIS

Table D.1 The presence of antibodies to the flue virus A (anti Ig G) in the blood serum of Caspian seals collected in 2020-2022 . (Scientific and Production Center of Microbiology and Virology of the RoK)

Sample	OD	S/N	Calculation of S/Nav ratio (sample/K-) S/N ≥ 0.6 – negative S/N < 0.6 – positive	
K+	0,2468	0,2997		
K-	1,3000	1,5788		
1 Seal/2020	0,4606	0,3657	≥ 0,6	Positive
2 Seal/2020	1,1235	0,8919	< 0,6	Negative
3 Seal/2020	1,1235	0,8919	< 0,6	Negative
4 Seal/2020	0,4997	0,3967	≥ 0,6	Positive
5 Seal/2020	0,9303	0,7386	< 0,6	Negative
6 Seal/2020	0,4882	0,3876	≥ 0,6	Positive
7 Seal/2020	1,1674	0,9268	< 0,6	Negative
8 Seal/2020	1,0237	0,8127	< 0,6	Negative
9 Seal/2020	0,9560	0,7590	< 0,6	Negative
10 Seal/2020	0,4554	0,3615	≥ 0,6	Positive
11 Seal/2020	1,2085	0,9594	< 0,6	Negative
12 Seal/2020	1,1779	0,9351	< 0,6	Negative
13 Seal/2020	1,0077	0,8000	< 0,6	Negative
1 Seal/2022	0,4222	0,3352	≥ 0,6	Positive
2 Seal/2022	0,2447	0,1943	≥ 0,6	Positive
3 Seal/2022	0,4843	0,3845	≥ 0,6	Positive
4 Seal/2022	0,4819	0,3826	≥ 0,6	Positive
5 Seal/2022	0,2620	0,2080	≥ 0,6	Positive
6 Seal/2022	0,1070	0,0849	≥ 0,6	Positive
7 Seal/2022	0,4112	0,3265	≥ 0,6	Positive
8 Seal/2022	0,2570	0,2040	≥ 0,6	Positive
9 Seal/2022	0,1324	0,1051	≥ 0,6	Positive
10 Seal/2022	0,1955	0,1552	≥ 0,6	Positive
11 Seal/2022	1,1760	0,9336	< 0,6	Negative
12 Seal/2022	1,1411	0,9059	< 0,6	Negative
14 Seal/2022	0,2088	0,1658	≥ 0,6	Positive
15 Seal/2022	0,7326	0,5816	≥ 0,6	Positive
16 Seal/2022	1,0095	0,8014	< 0,6	Negative
17 Seal/2022	0,6439	0,5112	≥ 0,6	Positive
18 Seal/2022	0,6068	0,4817	≥ 0,6	Positive
19 Seal/2022	0,2872	0,2280	≥ 0,6	Positive
20 Seal/2022	0,3300	0,2620	≥ 0,6	Positive
21 Seal/2022	0,6112	0,4852	≥ 0,6	Positive
22 Seal/2022	0,4861	0,3859	≥ 0,6	Positive
23 Seal/2022	0,0940	0,0746	≥ 0,6	Positive
24 Seal/2022	0,3296	0,2617	≥ 0,6	Positive
25 Seal/2022	0,2514	0,1996	≥ 0,6	Positive

Table D.2 The presence of antibodies to the carnivorous plague virus (CDV IgG ELISA TEST) in the blood serum of Caspian seals collected in 2019-2023 . (Scientific and Production Center of Microbiology and Virology of the RoK)

Sample	Reader	Results (U)	Interp.
1 Seal/PR/19	0,1679	3	Negative
3 Seal/PR/19	0,0648	1	Negative
4 Seal/PR/19	0,0746	2	Negative
5 Seal/PR/19	0,5299	11	Positive
1 Seal/PR/20	0,0609	1	Negative
2 Seal/PR/20	0,0793	1	Negative
3 Seal/PR/20	0,0857	2	Negative
4 Seal/PR/20	0,2884	5	Negative

Sample	Reader	Results (U)	Interp.
5 Seal/PR/20	0,3344	6	Equivocal
6 Seal/PR/20	0,2298	4	Negative
7 Seal/PR/20	0,1419	3	Negative
8 Seal/PR/20	0,1072	2	Negative
9 Seal/PR/20	0,1129	2	Negative
10 Seal/PR/20	0,2823	5	Negative
11 Seal/PR/20	0,0983	2	Negative
12 Seal/PR/20	0,0780	1	Negative
13 Seal/PR/20	0,1677	3	Negative
01/Seal/KU/2022	0,5615	10	Positive
02/Seal/KU/2022	0,2248	4	Negative
03/Seal/KU/2022	0,1838	3	Negative
04/Seal/KU/2022	0,1364	2	Negative
05/Seal/KU/2022	0,3432	6	Equivocal
06/Seal/KU/2022	0,1505	3	Negative
07/Seal/KU/2022	0,4496	8	Positive
08/Seal/KU/2022	0,4560	8	Positive
09/Seal/KU/2022	absent	absent	-
10/Seal/KU/2022	0,4205	8	Positive
11/Seal/KU/2022	0,0499	1	Negative
12/Seal/KU/2022	0,1175	2	Negative
14/Seal/KU/2022	0,5963	11	Positive
15/Seal/KU/2022	0,5155	9	Positive
16/Seal/KU/2022	0,1247	2	Negative
17/Seal/KU/2022	0,2302	4	Negative
18/Seal/KU/2022	0,0504	1	Negative
19/Seal/KU/2022	0,2706	5	Negative
20/Seal/KU/2022	0,2129	4	Negative
21/Seal/KU/2022	0,3012	5	Negative
22/Seal/KU/2022	0,1114	2	Negative
23/Seal/KU/2022	0,1172	2	Negative
24/Seal/KU/2022	0,2678	5	Negative
25/Seal/KU/2022	0,0863	2	Negative
26/Seal/KU/2022	0,0818	1	Negative
1 Seal/PR/23	0,1156	2	Negative
2 Seal/PR/23	0,1445	3	Negative
3 Seal/PR/23	0,3321	7	Equivocal
4 Seal/PR/23	0,5989	12	Positive
5 Seal/PR/23	0,1006	2	Negative
6 Seal/PR/23	0,3867	8	Positive
7 Seal/PR/23	0,1692	3	Negative
8 Seal/PR/23	0,3031	6	Equivocal
9 Seal/PR/23	0,1713	3	Negative
10 Seal/PR/23	0,0555	1	Negative
11 Seal/PR/23	0,2065	4	Negative
12 Seal/PR/23	0,1262	3	Negative
13 Seal/PR/23	0,1054	2	Negative
14 Seal/PR/23	0,0914	2	Negative
15 Seal/PR/23	0,0895	2	Negative
16 Seal/PR/23	0,1262	3	Negative
17 Seal/PR/23	0,0727	1	Negative
18 Seal/PR/23	0,1116	2	Negative
19 Seal/PR/23	0,1156	2	Negative
20 Seal/PR/23	0,0548	1	Negative
21 Seal/PR/23	0,0888	2	Negative
22 Seal/PR/23	0,1127	2	Negative
23 Seal/PR/23	0,0784	2	Negative
24 Seal/PR/23	0,2577	5	Negative

Table D.3 Identification of virus neutralizing antibodies in blood serum of Caspian seals collected in 2020-2022. (Scientific and Production Center of Microbiology and Virology of the RoK)

ID of serum	Volume (µl)	Slot	Serum dilution and results						Activity in four repetitions
			1:2	1:4	1:8	1:16	1:32	1:64	
01/Seal/KU/2022	1000	1	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
02/Seal/KU/2022	1000	2	-- --	-- --	-- -+	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:4,
03/Seal/KU/2022	1000	3	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
04/Seal/KU/2022	1000	4	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
05/Seal/KU/2022	1000	5	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
06/Seal/KU/2022	1000	6	-- --	-- --	++ ++	++ ++	++ ++	++ ++	1:4, 1:4, 1:4, 1:4,
07/Seal/KU/2022	1000	7	-- --	-- --	+- ++	++ ++	++ ++	++ ++	1:8, 1:4, 1:8, 1:8,
08/Seal/KU/2022	1000	8	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
09/Seal/KU/2022	1000	9	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
10/Seal/KU/2022	1000	10	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
11/Seal/KU/2022	1000	11	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
12/Seal/KU/2022	1000	12	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
14/Seal/KU/2022	1000	13	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
15/Seal/KU/2022	1000	14	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
16/Seal/KU/2022	1000	15	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
17/Seal/KU/2022	1000	16	-- --	-- --	++ ++	++ ++	++ ++	++ ++	1:4, 1:4, 1:4, 1:4,
18/Seal/KU/2022	1000	17	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
19/Seal/KU/2022	1000	18	-- --	-- --	-+ -+	++ ++	++ ++	++ ++	1:4, 1:8, 1:4, 1:8,
20/Seal/KU/2022	1000	19	-- --	-- --	+- --	++ ++	++ ++	++ ++	1:4, 1:8, 1:8, 1:8,
21/Seal/KU/2022	1000	20	-- --	-- --	+- +-	++ ++	++ ++	++ ++	1:4, 1:8, 1:4, 1:8,
22/Seal/KU/2022	1000	21	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
23/Seal/KU/2022	1000	22	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
24/Seal/KU/2022	1000	23	-- --	-+ --	+- --	++ ++	++ ++	++ ++	1:4, 1:8, 1:4, 1:8,
25/Seal/KU/2022	1000	24	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
26/Seal/KU/2022	1000	25	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
05/Seal/PR/2020	500	26	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,
06/Seal/PR/2020	500	27	-- --	-- --	-+ --	++ ++	++ ++	++ ++	1:8, 1:4, 1:8, 1:8,
07/Seal/PR/2020	500	28	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
09/Seal/PR/2020	500	29	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
10/Seal/PR/2020	500	30	-- --	-- --	-- --	++ ++	++ ++	++ ++	1:8, 1:8, 1:8, 1:8,

ID of serum	Volume (µl)	Slot	Serum dilution and results						Activity in four repetitions
			1:2	1:4	1:8	1:16	1:32	1:64	
12/Seal/PR/2020	500	31	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative
Positive, control			-- --	-- --	-- --	-- --	+- --	++ ++	1:16, 1:32, 1:32, 1:32,
Negative, control			++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	negative

Note: *«+» – CE in a cell culture monolayer.*
«-» – No CE in a cell culture monolayer, i. e. serum neutralizes the virus.

APPENDIX E PHOTOGRAPHIC MATERIALS

I. Seal capturing

Sand island KT5 (13.11.2023)

Sand island KT6, approaching boat (13.11.2023)

Seal capturing at sand island KT6 (13.11.2023)

II. Seal Surveys

Weighing a seal

Measurement, chest circumference

Age identification by claws

Wool sampling

Collecting an eye swab

Collecting a nasal swab

Collecting a vibrissa sample

Collecting blood from the hind flipper

Installing a radio transmitter

Releasing a seal with a satellite radio transmitter

Ultrasound examination of the fetus and fat accumulation