

Supplementary information

Exposure of wild Caspian seals (*Pusa caspica*) to parasites, bacterial and viral pathogens evaluated via molecular and serological assays

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Supplementary Table 1. Sampling locations and dates.

Year	Month	Location	Number of live sampled seals	Number of necropsied seal carcasses
2007	April	Seashore along Kalamkas oil field (45°25' N, 051°47' E)	-	8
	October	South-West isles (46°45' N, 51°38' E)	3	-
	November	Rybachy isle, Kulaly group (44°76' N, 50°37' E)	9	-
2008	November	Rybachy isle, Kulaly group (44°76' N, 50°37' E)	13	-
2009	November	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	7	1
2010	May	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	-	1
	October	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	18	-
2011	April	Durnev isles, Komsomolets Bay (45°30' N, 52°37' E)	46	-
2012	March	Bautino spit, Cape Urduke (44°23' N, 50°14'E)	-	6
	October	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	21	1
2013	May	Bautino spit (44°28'54" N, 50°14'28" E)	-	1
	November	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	8	-
2014	April	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	3	-
		Bautino spit (44°28'54" N, 50°14'28" E)	-	-
2015	April	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	1	6
		Aqtau (43°39' N, 51°09' E)	-	1
2016	May	Prorva artisanal isle (46°00' N, 53°04' E)	23	1
	October	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	20	-
	April	Komsomolets Bay	-	1
2017	April	Bautino spit (44°28'54" N, 50°14'28" E)	-	10
	October	Kendirli isles, Kazakh Bay (42°44' N, 52°32' E)	5	-
Total			177	37

Supplementary Table 2. Caspian seal health survey sample sizes, by sex, age class and sample type (live seals).

Year of sampling	Number of live sampled seals	Sex		Age class			Swab samples					Other samples	
		Male	Female	Juvenile	Sub-adult	Adult	Buccal	Nasal	Conjunctival	Rectal	Urogenital	Serum	Faeces
2007	12	4	4	8	1	1	12	12	0	12	0	12	0
2008	13	7	6	6	5	2	13	13	0	13	13	13	0
2009	7	3	4	1	3	3	7	3	7	7	7	0	0
2010	18	3	15	0	2	16	18	18	18	18	18	3	1
2011	46	34	11	0	4	41	37	30	0	37	37	24	0
2012	21	8	13	2	2	16	21	21	0	21	21	10	0
2013	8	3	5	4	2	2	8	8	8	8	8	8	0
2014	3	1	2	1	2	0	3	3	0	3	3	3	0
2015	1	1	0	0	1	0	1	1	1	1	1	1	5
2016	43	18	22	3	3	27	43	43	0	43	43	20	0
2017	5	1	4	1	0	4	5	5	0	5	0	3	20
Total	177	83	86	26	25	112	168	157	34	168	151	97	26

Supplementary Table 3. Amplification primers for genes of the specified virus species and families screened in Caspian seal samples.

Target gene	Primer sequence references	Expected product (b.p.)
M gene of influenza A virus ¹	CU-MF: 5'-TGATCTTCTTGAAAAATTGCG-3' CU-MR: 5'-TGTTGACAAAATGACCATCG-3'	279
NS gene of influenza B virus ²	NS1: 5'- ATG GCC ATC GGA TCC TCA AC-3' NS2: 5'- TGT CAG CTA TTA TGG AGC TG-3'	240
P gene of morbilliviruses ³	UP-P1:-5' ATGTTTATGATCACAGCGGT-3' UP-P2: -5' ATTGGGTTGCACCACTTGTC-3'	429
gD gene [glycoprotein D] of phocid herpesvirus type 1 (PhHV-1) ⁴	gD1: 5' - GAAGTTCGGTATGT[A/T]AC-3' gD2: 5' - TTAGGGTTAAGTAG[T/C]AG -3'	290
L gene of paramyxoviruses (Pan-paramyxovirus primer) ⁵	PMX1: 5' - GARGGIYIITGYCARAARNTNTGGAC - 3' PMX2: 5' - TIAYIGCWATIRIYTGRTRTCNCC -3'	132
L gene of paramyxoviruses (Pan-paramyxovirus primer) ⁶	PAR F1:-5'GAAGGITATTGTCAIAARNTNTGGAC-3' PAR F2:-5'GTTGCTTCAATGGTTCARGGNGAYAA-3'	600
poly L gene of coronaviruses (Pan-coronavirus primer) ⁷	Cor-FW:- 5'ACWCARHTVAAYYTNAARTAYG-3' Cor-RW:- 5' TCRCAYTTDGGRTARTCCC-3'	251
pol gene of adenoviruses ⁸	polFouter [5'-TNMGNGGNGGNGMGNTGYTAYCC-3'] polRouter [5'-GTDGCRAANSHNCCRTABARNGMRTT-3'] - for the 2 nd round polFinner (5'-GTNTWYGAYATHTGYGGHATGTAYGC-3' polRinner (5'-CCANCCBCDRTRTGNARNGTRA-3').	318-325

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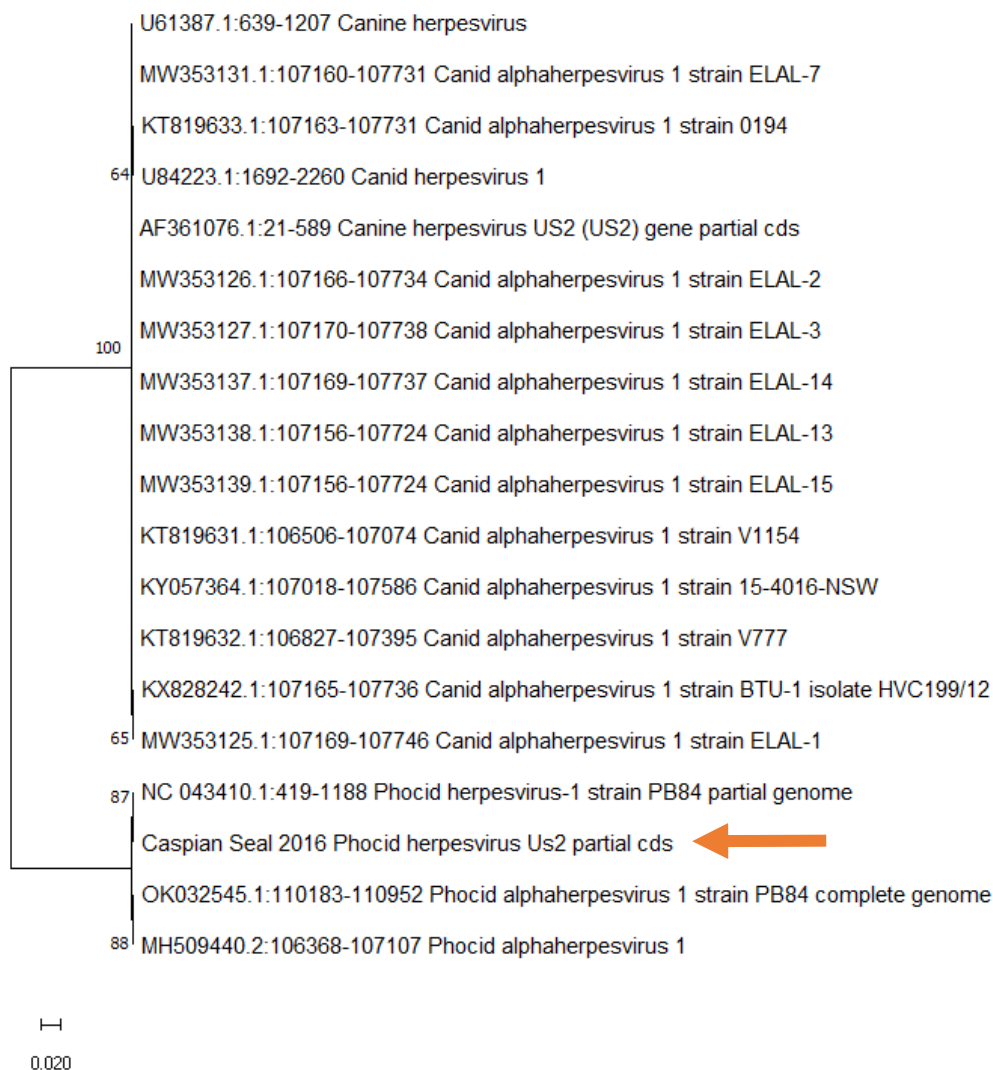
Supplementary Table 4. Thermocycling parameters for PCR with different virus screening primers.

Primers	Denaturation		Annealing		Elongation	
	t°C	duration	t°C	duration	t°C	duration
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
gD1/ gD2	94°C	30 sec	45°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
polFouter/ polRouter	94°C	30 sec	46°C	1 min	72°C	1 min
polFinner/ polRinner	94°C	30 sec	46°C	1 min	72°C	1 min

Supplementary Table 5. Sequence identity between Caspian seal P gene amplicon sequences.

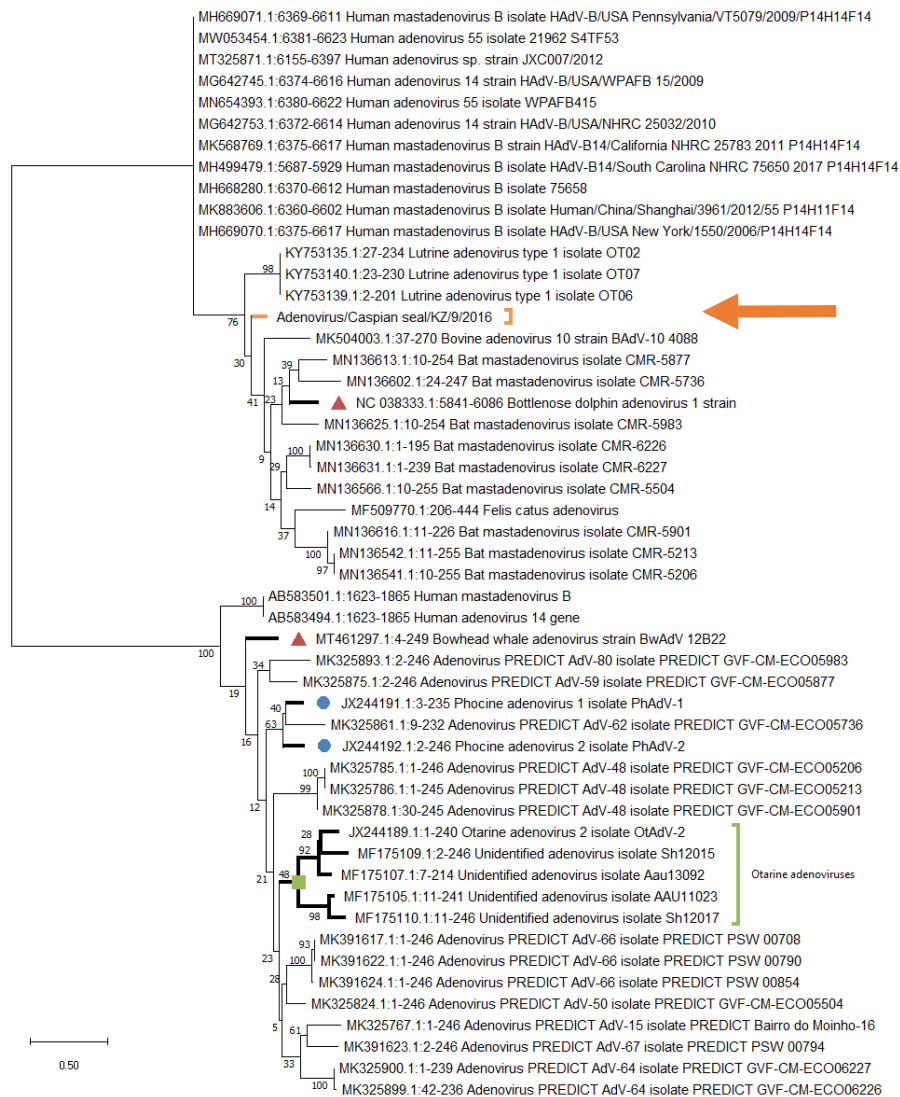
Strain	1	2	3	4	5	6	7	8
1 1BN CDV.sam contig 1 (2008 whole genome P gene sequence)	-							
2 P gene CDV 2008 (RT-PCR amplicon)	1.00000	-						
3 baku4 ^{1,2}	0.99502	0.99441	-					
4 baku5 ^{1,2}	0.99751	0.99721	0.99751	-				
5 kz1 ^{1,2}	0.99504	0.99441	1.00000	0.99751	-			
6 kz2 ^{1,2}	0.99504	0.99441	1.00000	0.99751	1.00000	-		
MN267066.1 Canine morbillivirus isolate PC00-21 complete genome								
7 (2000 outbreak) ³	0.99504	0.99441	1.00000	0.99751	1.00000	1.00000	-	
HM046486.1 Canine distemper virus strain Phoca/Caspian/2007								
8 complete genome	0.96526	0.96927	0.97015	0.96766	0.97022	0.97022	0.97022	-

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Supplementary Figure 1. Maximum Likelihood phylogenetic tree for partial sequences of the alphaherpesvirus type 1 Us2 gene.

Arrow indicates the position of the Caspian seal 2016 isolate. The tree was generated using the Tamura 3 parameter distance with 1000 bootstrap replicates.



Supplementary Figure 2. Maximum Likelihood phylogenetic tree for partial sequences of the Adenovirus pol gene.

Arrow indicates the position of the Caspian seal 2016 isolate. Position of isolate derived from other marine mammals highlighted by symbols. The tree was generated using the Tamura 3 parameter + G distance with 1000 bootstrap replicates.

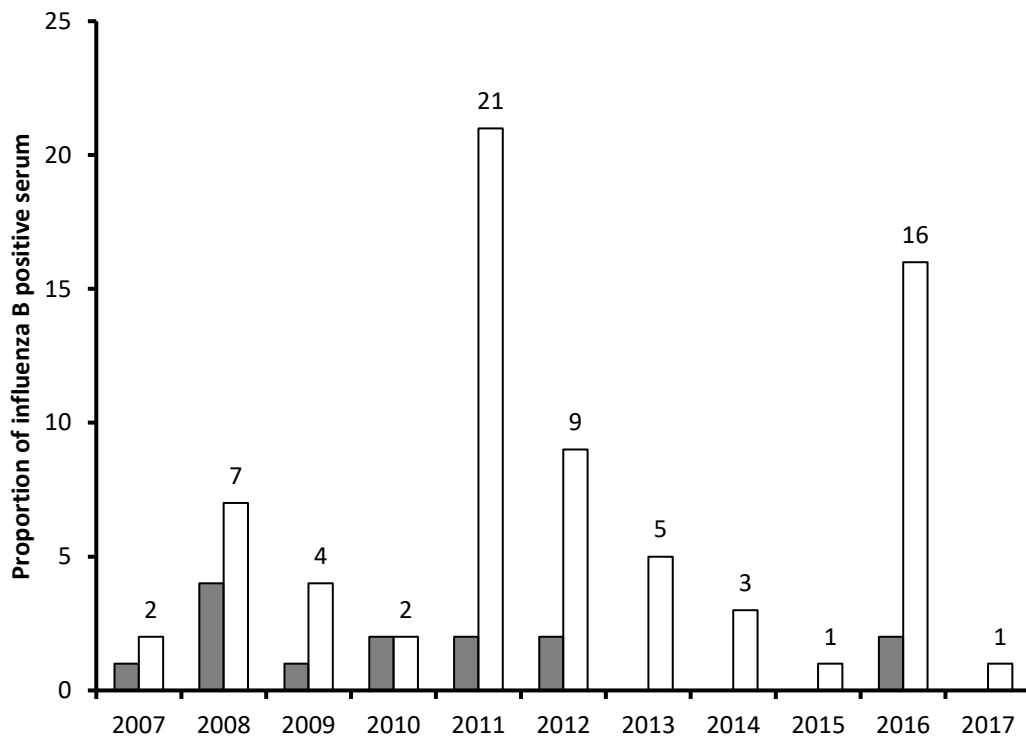
Supplementary Figure 2. Seal pathologies and injuries.



Supplementary Figure 1A – Caspian seal showing evidence of keratopathy on cornea.



Supplementary Figure 1B – Juvenile Caspian seal with suspected predator bite excising skin and blubber from the dorsal area between the scapulars and tail.



Supplementary Figure 3. Proportion of seals with antibodies against influenza B virus.

Caspian seal serum samples were tested with the hemagglutination inhibition test for the presence of antibodies against hemagglutinins of the Influenza B virus. White bars – total sample size, grey bars - number of sera with antibodies against the influenza B virus.

Supplementary Table 6. Age categories of Caspian seals testing positive for selected pathogens via PCR and serological tests. Grey shading highlights positive detections.

Season, year	PCR										IgG ELISA										HI test					LMAT		
	Juv		Sub-adult		Adult		Juvenile				Sub-adult				Adult				Adt		Juv	SA	Adt	Juv.	S-A	Adt		
	CDV		IAV		PhHV-1	AdV	CDV	CAV	CCV	CHV	CDV	CAV	CCV	CHV	CDV	CAV	CCV	CHV	TG	HW	IAV	IAV	IBV		Leptospira			
Autumn, 2007	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Autumn, 2008	4	1	0	0	0	0	1	2	0	5	0	1	0	2	0	0	0	0	0	0	0	0	3	1	0	2	1	1
Autumn, 2009	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	2	0	0	0	0	0	0	1	0	0	0
Autumn, 2010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	0	0
Spring, 2011	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	9	0	22	0	0	4	2	0	1	1	0	0	3
Autumn, 2012	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	9	1	1	1	0	1	0	1	0	0	0
Autumn, 2013	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Spring, 2014	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Spring, 2016	0	0	0	0	3	0	0	0	1	1	0	0	0	1	2	1	2	7	1	0	2	2	0	0	2	0	0	0
Autumn, 2016	0	0	1	3	2	5	0	0	0	1	0	1	0	1	1	1	0	6	0	0	1	1	0	0	0	0	0	0

Abbreviations: Adt – Adult; CAV - Canine adenovirus; CCV - Canine coronavirus; CDV - Canine distemper virus; CHV - Canine herpesvirus; HI - Hemagglutination inhibition test; HW – Heartworm; IAV - Influenza A virus; IBV - Influenza B virus; Juv – Juvenile; LMAT – Leptospira microscopic agglutination test; PhHV - Phocine herpesvirus; SA - Sub-Adult; TG - Toxoplasma gondii.

Supplementary Table 7. Evaluation of seasonal differences in prevalence viral pathogens from RT-PCR and PCR tests of Caspian seal swab samples.

Type of test	Pathogens	Test results	Spring (Prevalence, %)	Autumn (Prevalence, %)	p-value (Fisher's exact test)
RT-PCR	Canine Distemper Virus	Positive	0	5 (10.0)	0.056
		Negative	49	45	
RT-PCR	Influenza A virus	Positive	0	4 (5.63)	0.57
		Negative	28	67	
PCR	Seal Herpesvirus PhHV-1	Positive	3 (8.33)	2 (4.76)	0.66
		Negative	33	40	
PCR	Adenovirus	Positive	0	5 (21.7)	1
		Negative	0	18	
Total		Positive	3 (2.65)	16 (8.60)	
		Negative	110	170	

Supplementary Table 8. Evaluation of seasonal differences in antibody prevalence for selected pathogens in Caspian seals sera.

Type of test	Pathogens	Test results	Spring (Prevalence, %)	Autumn (Prevalence, %)	p-value (Fisher's exact test)
Ig ELISA	Canine Coronavirus	Positive	3 (6.67)	1(3.70)	1
		Negative	42	26	
	Canine Distemper Virus	Positive	3 (6.67)	3(10.3)	0.67
		Negative	42	26	
	Canine Adenovirus	Positive	10 (22.2)	8 (28.6)	0.58
		Negative	35	20	
	Canine Herpes Virus	Positive	35	32	0.12
		Negative	0	4	
	Influenza A virus	Positive	6 (15.0)	2 (5.56)	0.27
		Negative	34	34	
	Toxoplasma gondii	Positive	1 (2.17)	1(3.12)	1
		Negative	45	31	
	Heartworm	Positive	0	1 (3.70)	0.38
		Negative	45	26	
HI	Influenza A virus	Positive	4 (9.30)	1 (5.56)	1
		Negative	39	17	
	Influenza B virus	Positive	4 (10.0)	10 (33.3)	0.03
		Negative	36	20	
ID Screen ELISA	Influenza A virus	Positive	21(41.2)	14(29.2)	0.29
		Negative	30	34	
	Total	Positive	87 (20.0)	73 (23.5)	-
		Negative	348	238	