TOXICOLOGICAL AND PARASITOLOGICAL ANALYSIS OF EGYPTIAN VULTURE SAMPLES FROM BULGARIA AND GREECE

LIFE+ PROJECT
"THE RETURN OF THE NEOPHRON"
LIFE10 NAT/BG/000152

VCF Vulture Conservation Foundation

Ορνιθολογική Ελληνική Ορνιθολογική Εταιρεία

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THE REPORT
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The report is based on a clinical results report provided by the Center for Analysis and Diagnosis of Wildlife, CAD (INFORME DE RESULTADOS 1891/EX/14 1929 a 1939/EX/14) and the great collaboration between the Vulture Conservation Foundation (VCF), the Center for Analysis and Diagnosis of Wildlife (CAD) and the leading partner of the LIFE+ project - the Bulgarian Society for the Protection of Birds / BirdLife Bulgaria.

In collaboration with:

CENTRO DE ANÁLISIS Y DIAGNÓSTICO DE LA FAUNA SILVESTRE
CAD
JUNTA DE ANDALUCIA
Consejería de Medio Ambiente

JUNTA DE ANDALUCIA
CONSEJERÍA DE MEDIO AMBIENTE Y ORDENACIÓN DEL TERRITORIO

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EXECUTIVE SUMMARY

The Egyptian Vulture is declining throughout most of its distribution range. There are numerous threats affecting the species in its breeding and wintering areas, but also along its migratory route. The use of poison in the nature (different kinds of pesticides) seems to be one of the main limiting factors, but surely not the only threat affecting the species, as they also face a lack of food, habitat loss, collisions with power lines, wind-turbines and others.

This study aims to assess the health condition of the Egyptian Vulture population in the Balkan Peninsula (Bulgaria and Greece); identify possible infections (bacteria or virus) and potential exposure to toxic substances or intoxications caused by heavy metals, pesticides and veterinary medicaments.

A total of 182 samples (36 blood samples for toxicology, and 146 samples from throat, cloaca, and eye for pathogen analysis) from a total of 49 individuals coming from different Egyptian Vulture territories from Bulgaria and Greece, were collected mainly from fledglings during 2012 and 2013, and send for analysis in 2014 to CAD (Center for Analysis and Diagnosis of Wildlife), Malaga, Spain.

For the infectious part, we analysed a long list of microorganisms (Salmonella spp, Campylobacter spp, Escherichia coli 0157, Chlamidia spp, Avian mycoplasma, Avian adenovirus, Avian circovirus, Newcastle, West Nile and Bordetella), all of them known as potential pathogens affecting birds of prey or Egyptian Vulture in particular. For their identification and quantification we used microbiological and PCR (Polymerase Chain Reaction) analyses. The results have revealed that these microorganisms did not affect the sampled individuals. Only very low concentrations of Newcastle were detected in most of the samples and in some we detected a low concentrations of Avian adenovirus and Avian circovirus by PCR. This means that these individuals had only a contact with these viruses (probably very common), but for sure were not suffering disease. It is important to be mentioned, that not all samples were in perfect condition - properly taken and stored, so this may have influenced the results.

Very complete toxicological analyses were performed. We analysed heavy metals (lead and cadmium), 270 different pesticide compounds, 21 anti-inflammatories and 137 antibiotics. All these compounds can be found in vulture food (coming from agricultural and veterinary practices) and have or might have negative effects on the Egyptian Vultures.

The results were surprisingly good; nearly all of them negative. We only detected a very small insignificant amount of Aspirin (Acetylsalicylic acid) in one group of samples taken from Greece.

The analyses performed suggest that the individuals sampled were in good health condition during the period of sampling – not affected by any pathogen or toxic substances. While this does not allow us to extrapolate to the whole of the Balkans, and for other periods of time, it suggests that wildlife disease and intoxication with heavy metals (including lead), and with toxic compounds from agriculture or veterinarian practices may not be a significant threat to Egyptian Vultures in Bulgaria and Greece.

1. SAMPLES DESCRIPTION

A total of 182 samples (36 blood samples for toxicology, and 146 samples from throat, cloaca, and eye for pathogen analysis) from a total of 49 individuals coming from different Egyptian Vulture territories from Bulgaria and Greece, were sent for analysis in Center for Analysis and Diagnosis of Wildlife (CAD).

- In order to reduce costs and increase efficiency all these samples were grouped groups, creating 12 pools of samples (Table 1). The samples were grouped/pools created following the geographical position of the Egyptian Vultures nests of the sampled individuals.
- Additional samples from one Spanish individual were included in this study by VCF as single group/pool, mostly because of technical reasons: to have opportunity to compare the results of the samples taken in Bulgaria and Greece with fresh samples (recently and properly taken). The costs for these analyses are not included into this project collaboration; they were covered by the VCF.

Table 1: List of samples grouped in 12 pools

CAD Identification	External Identification	Origen	Samples	Conservation Condition
1891/EX/14	Ring 9070115	Spain - AMUS & VCF	Blood (1)	EDTA
			Cloaca (3)	Amies
			Ocular (1)	Dry swab
			Oropharynx (1)	Dry swab
1929/EX/14	BG01 (juvenile)	Bulgaria	Blood (2)	Heparin
			Cloaca (2)	Stuart
			Ocular (2)	Dry swab
			Oropharynx (2)	Dry swab
1930/EX/14	BG02 (juvenile)	Bulgaria	Blood (1)	Heparin
			Cloaca (1)	Stuart
			Ocular (1)	Dry swab
			Oropharynx (1)	Dry swab
1931/EX/14	BG03 (juvenile)	Bulgaria	Blood (1)	Heparin
			Cloaca (2)	Stuart
			Ocular (2)	Dry swab
			Oropharynx (2)	Dry swab
1932/EX/14	BG04 (juvenile)	Bulgaria	Blood (5)	Heparin
			Cloaca (6)	Stuart
			Ocular (6)	Dry swab
			Oropharynx (6)	Dry swab
1933/EX/14	BG05 (juvenile)	Bulgaria	Blood (8)	Heparin
			Cloaca (8)	Stuart
			Ocular (8)	Dry swab
			Oropharynx (8)	Dry swab
1934/EX/14	BG06 (juvenile)	Bulgaria	Blood (2)	Heparin
			Cloaca (6)	Stuart
			Ocular (7)	Dry swab
			Oropharynx (8)	Dry swab
1935/EX/14	BG07 (juvenile)	Bulgaria	Blood (6)	Heparin
			Cloaca (9)	Stuart
			Ocular (9)	Dry swab
			Oropharynx (10)	Dry swab
1936/EX/14	GR01 (juvenile)	Greece	Blood (5)	Heparin
			Cloaca (7)	Stuart
			Ocular (7)	Dry swab
			Oropharynx (7)	Dry swab
			Nasal secret (1)	Dry swab
1937/EX/14	GR02 (juvenile)	Greece	Blood (3)	Heparin
			Cloaca (3)	Stuart
			Ocular (3)	Dry swab
			Oropharynx (3)	Dry swab
1938/EX/14	GR03 (juvenile)	Greece	Blood (2)	Heparin
			Cloaca (2)	Stuart
			Ocular (2)	Dry swab
			Oropharynx (2)	Dry swab
1939/EX/14	GR04 (juvenile)	Greece	Blood (1)	Heparin
			Cloaca (1)	Stuart
			Ocular (1)	Dry swab
			Oropharynx (1)	Dry swab

2. CONSIDERATIONS

- Samples were taken between 2012 and 2013. Information is taken from the general info table provided.
- As a control and without additional costs VCF included samples taken from a juvenile Egyptian Vulture found weak in the field (Spain), after the recovery in AMUS the bird was released (September 2014).
- In the period between taking the samples and sending to CAD, most of the samples were kept cooled, although some of them have been frozen for several hours.
- The requested analyses were made using the pool method (grouping individual blood samples or swabs), regardless of the sampling date. The swabs pools were made mixing the samples into salted solution, in the minimum volume required for performing the analysis.
- Some requested analyses have been performed knowing that the storage medium is not the most appropriate (eg heparin for PCR analysis, may interfere with the method), and there is not enough information about the survival of microorganisms after a long period frozen or cooled swabs. Ideally, swab samples must be analysed immediately in the case of microbiology or preserved at very low temperatures (-80). Samples taken in AMUS (vulture with ring 9070115) were analysed 24 hours after the sampling and were cooled until the arrival in CAD.
- The analysis of pesticides, antibiotics, zinc and cadmium in blood may not reflect the possible concentration in the body because they are compounds whose target tissue is the kidney/liver mainly.

These considerations might have influenced the results obtained!

3. DESCRIPTION OF ANALYSES

3.1. Analyses requested

From blood samples (heparin)

- Anti-inflammatories
- Antibiotics
- Pesticides
- Lead
- Zinc
- Cadmium
- Avian adenovirus (PCR)
- Avian mycoplasma (PCR)
- Avian circovirus (PCR)
- Chlamydia (PCR)
- Newcastle (PCR)

Cloaca swabs (all in Stuart medium, except the one coming from AMUS in Amies)

- Salmonella spp
- Campylobacter spp
- Escherichia coli O157

Oropharynx swabs (dray)

- Chlamydia spp (PCR)
- Avian mycoplasma (PCR)
- West Nile (PCR)

- Newcastle (PCR)
- Bordetella (PCR)

Ocular swabs (dray):

- Avian mycoplasma (PCR)

3.2. Microbiological analysis

- Salmonella, Campylobacter and Escherichia coli O157

3.2.1. Salmonella spp

Salmonella analysis is carried out following the UNE-EN_ISO_6579:2003 = 2003 "Horizontal method for the detection of Salmonella spp" (8, 10, 11, 12).

- Briefly, the method includes a first step of bacteria growing in order to detect even low numbers of these bacteria or recover them if damaged. In continuation is the cultivation in semisolid medium specific for theirs growing and after in solid medium where its possible presence if confirmed through biochemical and serological analysis.

3.2.2. Campylobacter spp

Campylobacter analysis are carried out following the UNE EN ISO 10272:1:2006: "Horizontal method for the detection and count of Campylobacter spp." (8, 9).

- Briefly, the method includes a step of enrichment in liquid medium with incubation at two different temperatures. Then we proceed to two specific solid mediums for growing and finally the presence of the colonies is confirmed by biochemical, microscopically or movement analyses.

3.2.3. Escherichia coli O157

The analysis of Escherichia coli O157 are carried out following the UNE EN ISO 16654:2001 "Horizontal method for the detection of Escherichia coli O157" (8, 13).

- Briefly, the method includes four successive stages, starting with the growing in liquid medium, separation and concentration by immunomagnetic particles coated with antibodies of E. coli O157. Then we proceed to the isolation in solid culture medium selective for the bacteria and finally theirs presence is confirmed by biochemical and anti-serum agglutination tests O157.

3.3. Microorganisms analysis by PCR (Polymerase Chain Reaction)

DNA extraction was performed using the kit High Pure PCR Template by Roche, following the manufacturer's instructions. For the analysis of Newcastle the kit High Pure Viral RNA Kit by Roche was used for extraction of the RNA.

All analyses were made using real-time PCR with probes and primers designed by the laboratory and previously validated.

During the extraction a negative control in all analyses was used, to ensuring the absence of contamination in samples, and as positive control an extract of pure microorganism to avoid fake negative due to protocol.

In all graphical results the sample placed in the first position is the negative control and the last is the positive control.

Some additional notes:

- In the analyses for avian mycoplasma, three different positive controls were used: *Mycoplasma synoviae, Mycoplasma gallisepticum* and mix of both. At the results graph can be seen an overlay of canals. Two parallel analyses were made because of the large sample quantity.
- In the analyses for *Newcastle* the negative control contains control RT one step so it presents the melting temperature at the results graph.

- *Avian adenovirus* (EDS): real-time PCR is used with probes Taqman. At the results graph is presented together with Bordetella.
- *Mycoplasma aviar* (MycoAV): real-time PCR with probes Taqman.
- *Chlamydia psittaci* (PSI): real-time PCR with probes Taqman
- *Newcastle* (NDV): RT-PCR one-step in real-time with Sybr Green and analysis of melting temperature.
- *Bordetella* (BORD): real-time PCR with probes Taqman. At the results graph is presented together with avian adenovirus.
- *Circovirus* (BFV): PCR real-time with Sybr Green and analysis of melting temperature.

3.4. Pesticides analyses in blood

The extraction was made following the protocol described by Zoun & Spierenburg 1989 (14).

- Briefly: sample is mixed with sodium sulphate and extracted with dichloromethane after which it is purified by passage through columns containing Bio-Beads ® SX as support. In continuation with rotary evaporator is concentrated and finally reconstituted with 0.5 ml dichloromethane, obtaining a suitable extract for analysis. **Quantification of pesticides by liquid chromatography (UPLC-MS/MS) and gas (GC-MS/MS).**

The methods used by the laboratory are following the Directive 2002/657/CE (22): Gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) and Liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Samples were analysed for 270 different compounds (Table 2).

3.5. Heavy metals (lead, zinc and cadmium) analyses in blood

The sample extraction is performed by taking 1 ml of the mixed of Triton and additional standard.

Determination of Lead and Cadmium is performed by Atomic Absorption Spectrometry coupled to graphite chamber, while the determination of zinc was supposed to be done by atomic absorption spectrometry flame.

3.6. Analyses for anti-inflammatories, antibiotics, anthelmintics, anticoccidials and antifungal in blood

For the anti-inflammatory analysis the extraction was acidified by addition of formic acid acetonitrile. The samples are mixed for 1 hour in rotary shaker and then centrifuged.

Analysis of antibiotics, anthelmintics, anticoccidials and antifungals is performed by addition of acetonitrile acidified with nitric acid using polytron. The samples are centrifuged, magnesium sulphate is added and mixed. An aliquot is taken of the supernatant that is evaporated and re-dissolved in methanol:formic.

The determinations of all compounds were performed using a quantitative method - low resolution Liquid Chromatography coupled to Tandem Mass Spectrometry and Triple Quadrupole detector. This methodology allows confirmation of a positive result. Calibration is performed in white matrix (biota). Samples were analysed for 21 anti-inflamatory compounds (Table 3) and 137 compounds of antibiotics, anthelmintics, anticoccidials and antifungals (Table 4).

Table 2: Analysed compounds (total of 270)

2-fenilfenol	Cymoxanil	Flubendiamide	Methomyl	Methoxyfenocide	S421
Abamectina	Cinidon-ethyl	Flucythrinate	Fludioxonil	Mevinphos	Silafluofen
Acephate	Cipermitrin	Disulfoton-sulfone	Flufenoxuron	Myclobutanil	Simazine
Acetamiprid	Cyproconazole	Disulfoton-sulfoxide	Fluquinconazole	Monotocrofos	Spinosad
Aclonifen	Cyprodinil	Diuron	Flurochloridone	Nitempiram	Spirodiclofen
Acrinatrina	Cyromazine	Dodina	Flusilazole	Norflurazon	Tau-fluvalinate
Aldicarb	Chlofentezine	Emamectin-benzoate	Flutolanil	Nuarimol	Tebuconazole
Aldicarb sulfone	Chlorantraniliprole	Endosulfan	Flutriafol	Ofurace	Tebufenozide
Aldicarb sulfoxide	Chlordane	Endosulfan beta	Folpet	Omethoate	Tebufenpyrad
Amitraz	Chlorfenapyr	Endosulfan sulfate	Fonophos	Oxadiyl	Tecnazene
Azaconazole	Chlorfenson	Endrin	Forchlorfenuron	Oxamyl	Teflubenzuron
Azadiractin	Chlorfenvinphos	Epoxiconazole	Formetanate	Oxidimeton-methyl	Tefluthrin
Azinphos-ethyl	Chloridazon	Esfenvalerate	Formotion	Oxyfluorfen	Terbufos
Azinphos methyl	Chlormephos	Spiromesifen	Phosalone	p,p'-DDD+o,p-DDT	Terbutilacina
Azoxystrobin	Chlorobenside	Epiroxamin	Phosphamidon	p,p'-DDE	Terbutryn
Benalaxyl	Chloropropilat	Ethiofencarb	Phosmet	p,p-DDT	Tetrachlorvinphos
Bendiocarb	Chlorpiryfos	Etiofencarb sulfone	Furalaxil	Paclobutrazol	Tetraconazole
Biphenyl	Chlorpiryfos methyl	Etiofencarb sulfoxide	Furathiocarb	Ethyl parathion	Tetradifon
Bifenox	Chlortaldimetil	Etion	Furmeciclox	Mathyl paratyon	Thiabendazole
Bifenthrin	Clortalonil	Etofenprox	Lindane	Pencycuron	Thiacloprid
Bioallethrin	Clothianidina	Ethofumesate	Hexaconazole	Penconazole	Thiamethoxam
Bitertanol	Chlozolinate	Ethoprophos	Hexythiazox	Pendimethalin	Thiodicarb
Boscalid	Coumaphos	Etoxazole	Hymexazol	Permethrin	Thiophanat-methyl
Bromacil	Deltamethrin	Ethoxyquin	Imazalil	Pymetrozina	Thiofanox
Bromophos ethyl	Demeton-s-methyl	Etridiazole	Imidacloprid	Piperonyl butoxide	Thiofanox sulfone
Bromophos methyl	Demeton-s-methylsulfone	Etrimfos	Indoxacarb	Pyraclostrobin	Thiofanox sulfoxide
Bromopropylate	Demetrin	Famoxadone	Iprodione	Pyrazophos	Tolclofos-methyl
Bromuconazole	Diazinon	Fenamiohos	Iprovalicarb	Pyridaben	Tolyfluanid
Bupirimato	Dichlobenil	Fenarimol	Isocarbofos	Pyridaphenthion	Transfluthrin
Buprofecin	Diclobutrazol	Fenazaquin	Isofenphos	Pyrifenox	Triadimefon
Butocarboxim	Dichlofenthion	Fenbuconazole	Isofenphos methyl	Pyrimethanil	Triadimenol
Butocarboximsulphoxid	Dichlofluanid	Fenbutatin oxide	Lambda cyhalothrin	Pirimicarb	Triazophos
Butoxicarboxim	Dichloran	Fenhexamid	Linuron	Pirimifos methyl	Trichlorfon
Butralin	Dichlorobenzamide	Fenitrothion	Lufenuron	Pyriproxyfen	Tricresyl-phosphate
Cadusafos	Dichlorvos	Fenmedifam	Malathion	Procymidone	Trifloxystrobin
Captafol	Dicofol	Fenoxycarb	Mecarbam	Procloraz	Triflumizole
Captan	Dicrotophos	Fenpyroximate	Mepanipyrim	Profenofos	Triflumuron
Carbaryl	Dieldrin	Fenpropathrin	Metaflumizone	Promecarb	Trifluralin
Carbendazim	Diethofencarb	Fenpropidine	Metalaxyl	Propachlor	Vamidothion
Carbofenotion	Diphenylamine	Fenpropimorph	Methamidophos	Propamocarb	Vinclozolin
Carbofuran	Difenoconazole	Fensulfothion	Methidation	Propargite	
Carbofuran-3-hydroxy	Diflubenzuron	Fenthion	Methiocarb	Propiconazole	
Cyanofenphos	Dimethoate	Phenthoate	Methiocarb-sulfone	Quinalphos	
Cycloate	Dimethomorph	Fipronil	Propoxur	Chuinomethionat	
Cyfluthrin	Diniconazole	Flonicamid	Prothiofos	Quinoxyfen	
Cyhexatin	Disulfoton	Metyocarb-sulfoxide	Methoxychlor I	Quintocene	

Table 3: Anti-inflammatory compounds analysed (21 in total)

Diclofenac; 3-hydroxy diclofenac; 3-hydroxy-4-methoxy diclofenac; 4,5-hydroxy diclofenac; 4-hydroxy diclofenac; 5-hydroxy diclofenac.	Oxyphenylbutazone	Meclofenamic acid
Tolfenamic acid	Acetylsalicylic acid	Metamizol
Carprofen	Niflimic acid	Piroxicam
Flunixin	Phenylbutazone	Carboxiibuprofen
Flunixin meglumine	Indomethacin	Flurbiprofen
Meloxicam	Naproxen	Suxibuzone
Ketoprofen	Flufenamic acid	Vedaprofen

Table 4: Antibiotics, anthelmintics, anticoccidials and antifungals analysed (137 compounds in total)

ANTIBIOTICS			
Quinolones	Betalactams	Macrolides	Sulfonamides / Sulfamides
Flumequine	Penicillins	Macrolides C14	Dapsone
Nalidixic acid	Penicillin G	Erythromycin	Sulfadoxine
Oxolinic acid	Penicillin V	Roxithromycin	Sulfaetidol
			Sulfaguanidine
Fluoroquinolones	Aminopenicilinas	Macrolides C15	Sulfamerazine
Cinoxacin	Amoxicilina	Tulathromycin	Sulfamethazine
Ciprofloxacin	Ampicilina		Sulfameter
Danofloxacin	Penicillins antiestafilococos	Macrolides C16	Sulfamethizole
Difloxacin	Cloxacilin	Aivlosina	Sulfamethoxazole
Enoxacin	Dicloxacilin	Spiramycin	Sulfamethoxypyridazine
Enrofloxacin	Nafcillin	Tilmicosin	Sulfamonomethoxine
Fleroxacin	Oxacillin	Tylosin	Sulfamoxola
Lomefloxacin	Cephalosporins	Josamycin	Sulfanitran
Marbofloxacin	Cefalexin	Tylvalosin	Sulfapyridine
Norfloxacin			Sulphaquinoxaline
Pefloxacin	Nitrofurans	Macrolides in tests	Sulfathiazole
Sarafloxacin	Furaltadone	Troleandomycin	Sulfatroxazol
	3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ)		Sulfisomidine
Lincosamides	Furazolidone	Inhibitors of the dihydrofolate reductase	Sulfisoxazole
Pirlimycin	3-amino-2-oxazolidinone (AOZ)	Trimethoprim	Sulfabenzamide
Lincomycin	Nitrofurantoin		Sulfacetamide
	Nitrofurazon	Imidazoles	Sulfacloropyridazine
Pleuromutilines		Dimetridazole	Sulfadiazine
Tiamulin	Phenicols	Ipronidazole	Sulfadimethoxine
Valnemulin	Chloramphenicol	Ipronidazole-hydroxid	Sulfadimidine
	Florfenicol	Metronidazole	
Tetracyclines	Tianfenicol	Metronidazole-hydroxid	
Chlorotetracycline		Ronidazole	
Demeclocycline	Others	Ternidazole	
Doxycycline	Lecomicin A1	Tinidazole	
Minocycline	Virginiamycin M1		
Oxytetracycline			
Tetracycline			
ANTHELMINTICS	**ANTICOCCIDIALS**	**ANTIFUNGALS**	
Albendazole	Amprolium	Natamycin	
Amino albendazole sulfone	Carnidazol		
Albendazole sulfone	Clazuril		
Albendazole sulfoxide	Decoquinate		
Amino flubendazole	Diclazuril		
Amino mebendazole	Halofuginone		
Avermectin B1a	Lasalocid		
Avermectin B1b	Lasalocid Na		
Cyclobendazole	Maduramycin		
Doramectin	Monesin		
Emamectin B1a	Monesin Na		
Eprinomectin B1a	Narasin		
Febantel	Narasin Na		
Fenbendazole	Nicarbazin		
Fenbendazole sulfone	Nigerizin		
Fenbendazole sulfoxide	Robenidin		
Flubendazole	Salinomycin		
Hydroxy flubendazole	Salinomycin Na		
Hydroxy mebendazole	Semduramycin		
Ivermectin B1a	Semduramycin Na		
Levamisole	Toltrazuril		
Mebendazole			
Oxfendazole			
Oxfendazole sulfone			
Oxibendazole			
Thiabendazole			
5-hydroxy tiabendazole			

4. RESULTS

Table 5: Summarized results

	ID CAD	1891/EX/14	1929/EX/14	1930/EX/14	1931/EX/14	1932/EX/14	1933/EX/14	1934/EX/14	1935/EX/14	1936/EX/14	1937/EX/14	1938/EX/14	1939/EX/14	% (n=12)
Samples	**Analysis**	**Ring 9070115**	**BG01**	**BG02**	**BG03**	**BG04**	**BG05**	**BG06**	**BG07**	**GR01**	**GR02**	**GR03**	**GR04**	
Blood	*NSAIDs*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**Acetylsalicylic acid 0,067 mg/kg**	Negative	Negative	**1/12**
Blood	*Antibiotics*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Blood	*Pesticides*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Blood	Heavy metals - Pb	22,88 µg/dL Exposure	Insufficient sample	7,24 µg/dL Background non toxic exposure	Insufficient sample	<2,5 µg/dL Background non toxic exposure	7,51 µg/dL Background non toxic exposure	<2,5 µg/dL Background non toxic exposure	6,10 µg/dL Background non toxic exposure	7,22 µg/dL Background non toxic exposure	<2,5 µg/dL Background non toxic exposure	2,76 µg/dL Background non toxic exposure	<2,5 µg/dL Background non toxic exposure	**1/12**
Blood	Heavy metals - Cd	<0,10 µg/dL Background non toxic exposure	Insufficient sample	<0,10 µg/dL Background non toxic exposure	Insufficient sample	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	<0,10 µg/dL Background non toxic exposure	**0/12**
Blood	Heavy metals - Zn	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	Insufficient sample	**No**
Blood	*PCR - Avian adenovirus*	Negative	**Weak Positive**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	Negative	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	Negative	Negative	Negative	Negative	Negative	**4/12**
Blood	*PCR - Avian mycoplasma*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Blood	*PCR - Avian circovirus*	Negative	**Weak Positive**	Negative	Negative	**Weak Positive**	**Weak Positive (extremely low)**	Negative	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive**	Negative	**7/12**
Blood	*PCR - Chlamydia*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Blood	*PCR - Newcastle*	**Weak Positive**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**12/12**
Cloaca swabs	Microbiology - Salmonella	**Positive**	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**1/12**
Cloaca swabs	Microbiology - Campylobacter	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Cloaca swabs	Microbiology - Escherichia coli O157	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Throat swabs	*PCR - Chlamydia*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Throat swabs	*PCR - Avian mycoplasma*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Throat swabs	*PCR - West Nile*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Throat swabs	*PCR - Newcastle*	**Weak Positive**	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**1/12**
Throat swabs	*PCR - Bordetella*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**
Eye swabs	*PCR - Avian mycoplasma*	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	**0/12**

4.1. Microbiological analysis (Salmonella, Campylobacter y Escherichia coli O157)

Summary table:

Sample condition	Microorganism	Result
Cloaca swabs	*Salmonella*	**1/12 ***
Cloaca swabs	*Campylobacter*	**0/12**
Cloaca swabs	*Escherichia coli O157*	**0/12**

- All samples from Bulgaria and Greece were negative.
- The only sample that has been positive is from the Egyptian Vulture (RING 9070115) whose samples were taken by VCF/AMUS in Spain. They were sent to the CAD and analysed immediately. This indicates that maybe the samples from Bulgaria and Greece were improperly taken or damaged during the conservation process, referring only to this microbiological analysis.

4.2. Microorganisms analyses by PCR (Polymerase Chain Reaction)

Summary table:

Sample condition	Microorganism	Result
Blood	*Avian adenovirus*	**5/12**
Blood	*Avian circovirus*	**7/12**
Blood	*Chlamydia*	**0/12**
Oropharynx swab		**0/12**
Blood	*Newcastle*	**12/12**
Oropharynx swab		**1/12**
Oropharynx swab	*West Nile*	**0/12**
Oropharynx swab	*Bordetella*	**0/12**
Oropharynx swab	*Avian mycoplasma*	**0/12**
Oropharynx swab		**0/12**
Blood		**0/12**

4.2.1. All individuals have negative result of the following microorganisms using real time PCR

Avian mycoplasma	Blood, Oropharynx swab, ocular swab
Chlamydia	Blood, Oropharynx swab
West Nile	Oropharynx swab
Bordetella	Oropharynx swab

4.2.2. Avian adenovirus: PCR blood samples

- in heparin (except Egyptian Vulture Ring 9070115, with EDTA).

The individuals with weak or very weak positive results (5/12) are the following:

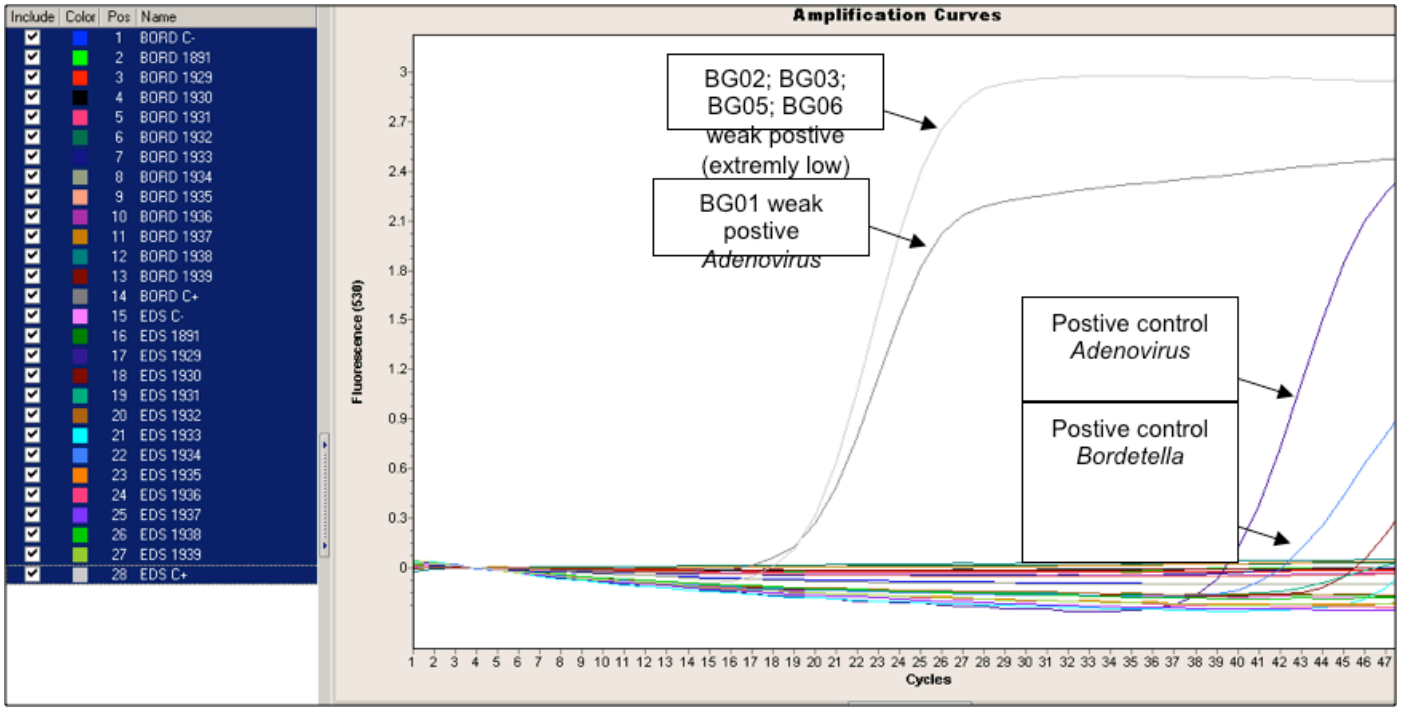
	1929/EX/14	1930/EX/14	1931/EX/14	1933/EX/14	1934/EX/14
	BG01	**BG02**	**BG03**	**BG05**	**BG06**
Territory_ID	RBG1/RDAJ1	NBUCHH1	RDAJ1	RPAT1; RGAB1; RKOV1; RPATE1; RPAT1; RGAB1; RGUR1	NBPRO1
Sampling date	27/07/2012 28/07/2013	24/07/2013	29/07/2012	28/07/2012; 27/07/2012; 04/08/2013; 31/07/2013; 02/08/2013	24/07/2013
Result	**Weak Positive**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**

The interpretation of the results (common amplification curves of Adenovirus and Bordetella analyses) was made from the graph obtained:

The real-time PCR for these studies were performed using Taqman probes. The graph increases correspond to DNA quantification of microorganisms studied (vertical axis Y, fluorescence 530 nm). Positive controls (in abscissa X, rise from cycle 17) presented.

When further controls starts rising, the lower concentration of DNA detected.

From about 45 cycles it is considered as background noise for any increase initiated.



- All samples were negative on Bordetella
- **Positive results on Adenovirus were detected in the samples groups/pools coming from Bulgaria (BG01, BG02, BG03, BG05 and BG06).**
- **The positive results are only showing that these individuals had contact with the virus but were not sick when the samples were taken.**

4.2.3. Avian circovirus (7/12): PCR – blood samples

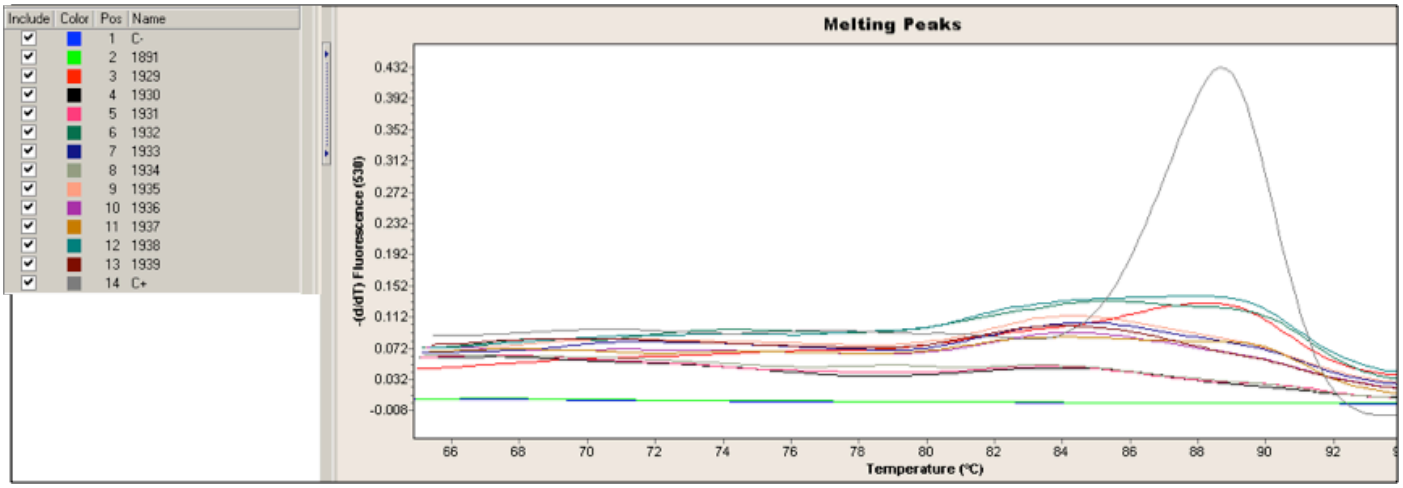
- in heparin (except Egyptian Vulture Ring 9070115, with EDTA).

The individuals with weak or very weak positive results (7/12) are the following:

	1929/EX/14	1932/EX/14	1933/EX/14	1935/EX/14	1936/EX/14	1937/EX/14	1938/EX/14
	BG01	BG04	BG05	BG07	GR01	GR02	GR03
Territory_ID	RBG1/RDAJ1	RDJA1; RKON1; RBIV1; RDJA1; RKON1	RPAT1; RGAB1; RKOV1; RPATE1; RPAT1; RGAB1; RGUR1	RDCH1; RCHA1; RTEP1; RSA1;	SEIT1; KAPS1	PALE; AGPA	KOMP3
Sampling date	27/07/2012 28/07/2013	26/07/2012; 28/07/2012; 29/07/2012; 28/07/2013	28/07/2012; 27/07/2012; 04/08/2013; 31/07/2013; 02/08/2013	26/07/2012; 03/08/2013; 01708/2013; 27/07/2013	19/07/2012; 07/08/2012; 11/09/2012; 07/08/2013	03/08/2012; 30/07/2013; 31/07/2013	05/08/2013
Result	**Weak Positive**	**Weak Positive**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive (extremely low)**	**Weak Positive**

Interpretation of the results (melting peaks - Avian circovirus) from the graph obtained:

The real-time PCRs for these studies were performed by using Sybr Green (flourofore) analysis and melting temperature. The fluorophore specifically binds DNA and specifically identifies the wanted fragment from the melting temperature. In the graph can be observed that the curve is associated with a particular temperature (in this case in psittacine circovirus 88,9ºC). The peak height indicates a clearly positive result. For the study was taken into account a deviation of ± 3 ° C due to the difference of a few nucleotides in raptors, thus avoiding loss of information when dealing with a non-psittacine species. As shown in the graph, considering the temperature deviation, the individuals listed in the table may contain very low concentrations, mostly extremely low for this virus.



- Positive results were obtained from samples pools from Bulgaria (BG01, BG04, BG05 and BG07) and from Greece (GR01, GR02 and GR03)
- The positive results are only showing that these individuals had contact with the virus but were not sick when the samples were taken.

4.2.4. Newcastle PCR blood samples and dry oropharynx swabs

- blood samples in heparin (except Egyptian Vulture ring 9070115, in EDTA)

The Individuals with weak positive or very weak positive results (12/12 in blood; 1/12 in oropharynx) are the following:

	1891/EX/14	1929/EX/14	1930/EX/14	1931/EX/14	1932/EX/14	1933/EX/14
	9070115	BG01	BG02	BG03	BG04	BG05
Territory_ID blood	VCF/AMUS	RBG1/RDAJ1	NBUCHH1	RDAJ1	RDJA1; RKON1; RBIV1; RDJA1; RKON1	RPAT1; RGAB1; RKOV1; RPATE1; RPAT1; RGAB1; RGUR1
Territory_ID oropharynx		RBG1				
Sampling date blood	23/09/2014	27/07/2012 28/07/2013	24/07/2013	29/07/2012	26/07/2012; 28/07/2012; 29/07/2012; 28/07/2013	28/07/2012; 27/07/2012; 04/08/2013; 31/07/2013; 02/08/2013
Sampling date oropharynx				29/07/2012; 27/07/2012	26/07/2012; 27/07/2012; 29/07/2012; 28/07/2013	
Results blood	Weak Positive	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)
Results oropharynx	Weak Positive	Negative	Negative	Negative	Negative	Negative

	1934/EX/14	1935/EX/14	1936/EX/14	1937/EX/14	1938/EX/14	1939/EX/14
	BG06	BG07	GR01	GR02	GR03	GR04
Territory_ID blood	NBPRO1	RDCH1; RCHA1; RTEP1; RSA1;	SEIT1; KAPS1	PALE; AGPA	KOMP3	NEAS2
Territory_ID oropharynx	NBPRO1 NBRAZ1; NBMAD1; NBPRO1					
Sampling date blood	24/07/2013	26/07/2012; 03/08/2013; 01/08/2013; 27/07/2013	19/07/2012; 07/08/2012; 11/09/2012; 07/08/2013	03/08/2012; 30/07/2013; 31/07/2013	05/08/2013	19/07/2012
Sampling date oropharynx	24/07/2013; 01/08/2012; 02/08/2012	26/07/2012; 29/07/2012; 03/08/2013; 01/08/2013; 27/07/2013	19/07/2012; 07/08/2012; 16/08/2012; 11/09/2012; 07/08/2013	03/08/2012; 23/07/2013; 31/07/2013		
Results blood	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)	Weak Positive (extremely low)
Results oropharynx	Negative	Negative	Negative	Negative	Negative	Negative

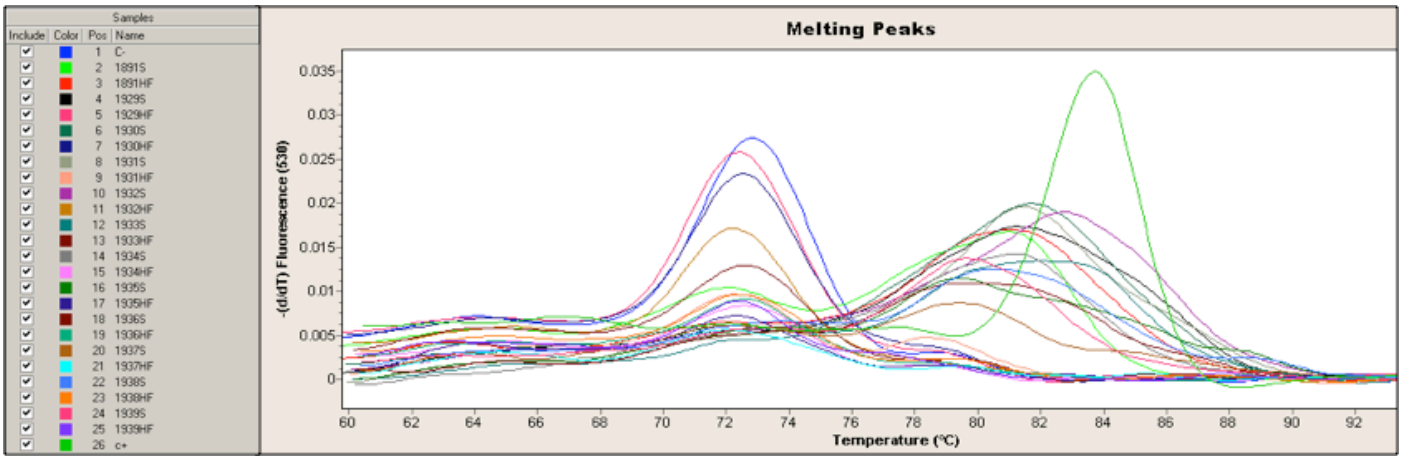
Interpretation of the results (melting peaks Newcastle) from the figure obtained:

The real-time PCR for these studies were performed by using SYBR Green (flouroforo) analysis and melting temperature. The fluorophore specifically binds to DNA and specifically identifies the fragment wanted from the melting temperature.

In this graph two groups of peaks can be observed:

- *Negative control: RT control contains one step at having melting temperature in the graph results.*
- *Positive control: melting curve associated with a given temperature (here 83,7ºC for NDV). The peak height indicates a clearly positive result. For the study was taken into account a deviation of ± 6 ° to keep possible point mutations of the virus.*

As can be seen in the graph, considering the temperature deviation, the individuals listed in the table may contain very low concentrations, mostly extremely low for this virus.



- **From all samples positive results were obtained (Bulgaria, Greece and the samples from the Spanish bird)**
- **The positive results from the samples taken from the Spanish bird are confirming that the sampling method and the conservation process did probably not affect the samples taken in Bulgaria and Greece.**
- **The positive results are only showing that these individuals had contact with the virus but were not sick when the samples were taken.**

4.3. Pesticides analyses in blood samples

- **All individuals resulted negative to pesticides detection analyses in blood samples in heparin** (ex cept individual with ring 9070115 in EDTA).

4.4. Antibiotics analyses in blood samples

- **All individuals resulted negative to antibiotics detection analyses in blood samples in heparin** (ex cept individual with ring 9070115 in EDTA).

4.5. Heavy metals analyses: lead, zinc and cadmium

- **Results obtained from the blood samples in heparin** (except individual with ring 9070115 in EDTA)

Condition of samples/individuals	Results		
	Lead	Zinc	Cadmium
Blood - heparin / groups BG01 to BG 07; GR01 to GR04	Insufficient sample BG01 and BG03	Insufficient sample *	Insufficient sample BG01 y BG03
	For the rest of the individuals concentrations ranging from <2,5 µg/dL to 7,51 µg/dL (no pathological background)		For the rest of the individuals is below the detection limit (<0,10 µg/dL)
Blood EDTA/ ring 9070115	**22,88 µg/dL (recent exposition)**		

* The amount of sample for all metals analysis was insufficient, so the zinc was not analysed. However, it is a metal that accumulates rapidly in kidney is very difficult to be detected in blood, even after recent exposure.

- Considering the insufficient sample material and also the fact that creating the pools (mixing the samples) might lower the metals concentration, we can say that no significant lead of cadmium intoxication was detected in the analysed individuals from Bulgaria and Greece.
- The high lead concentration detected in the individual with ring number 9070115 (from Spain) is due to

the lead intoxication during its recovery into the AMUS center.

4.6. NSAIDs analyses in blood samples

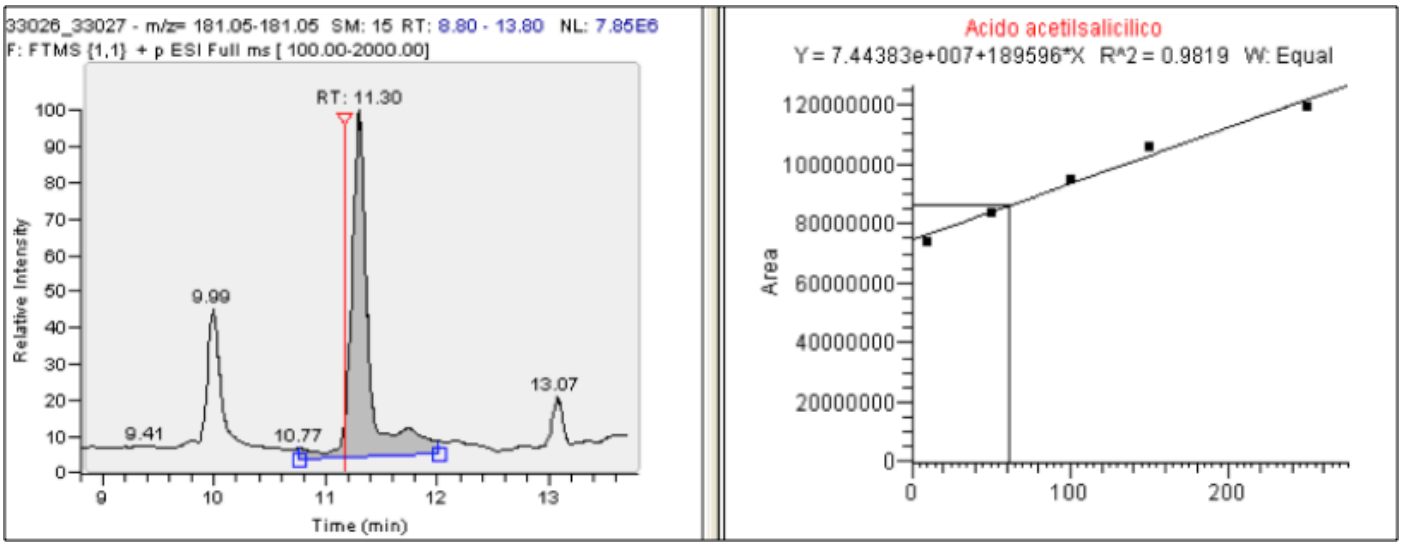
- All NSAIDs results of the on blood samples remitted (heparin, except individual ring 9070115 in EDTA) are negative except the following results:

GR02 group: acetylsalicylic acid (0.067 mg / kg) detected from the pool of blood samples collected on following days and territories:

03/08/2012	PALE
30/07/2013	PALE
31/07/2013	AGPA

- After the first preliminary results the colleagues from the Hellenic Ornithological Society have identified that the areas where these samples were taken (Greece), the Aspirin (acetylsalicylic acid) use is very common for treatment of turkeys and chickens.
- We can say that this very low consecration of acetylsalicylic acid is not affecting the halt condition of these individuals.

Spectrum results shown below:



5. SUMMARY OF THE RESULTS/INTERPREATION

5.1. Preliminary considerations

For correct interpretation of the results should be considered the condition of the samples, time between sampling and arrival into the lab, samples conservation, preservation temperature and maintenance (cooling / freezing). Furthermore it is also important to consider that the analyses were made using the pool method (blood, cloaca, oropharynx), so potential dilution effects or fake negative results are not discarded.

The period of resistance during the cooling / freezing is deferent for each microorganism. There are no published data on the viability of microorganisms studied after this period of time between the taking and analysis of the samples (1 or 2 years) time, so that the obtained negative results do not exclude a possible infection or contamination with any of the compounds analysed.

The only exceptions are the samples from the Egyptian Vulture with ring 9070115 that were taken by VCF/AMUS and sent to the laboratory immediately. The conservation means were suitable for the relevant analysis.

Conclusion: the results obtained in this study are applicable only on the pool of samples included in the study and the sampling and conservation conditions before their delivery to the CAD need to be considered.

All results have been included in an overview table (section 5).

5.2. Microbiological analyses

- Salmonella, Escherichia coli O157 and Campylobacter from cloaca samples (swabs)

In this study we have included three bacteria because of its importance in birds - domestic and wild (7, 23) and considering the importance for other animal species that can live with or have contact with the Egyptian Vulture. These bacteria can act as primary pathogens or as opportunistic pathogens mainly immunosuppressive states or disruption of normal enteric microbiota (24).

- **Only Salmonella was detected in the Egyptian vulture with ring 9070115 samples.**
- False negative results are possible from the cloaca samples analysed because of reasons stated in paragraph 6.1.

5.3. Microorganisms PCR analyses (polymerase chain reaction).

The analyses were done using real-time PCR, to an important group of bacteria and viruses:

- Avian adenovirus in blood
- Avian mycoplasma in blood, oropharynx and ocular samples
- Avian circovirus in blood
- Chlamydia in blood and oropharynx
- Newcastle in blood and oropharynx
- West Nile in oropharynx
- Bordetella in oropharynx

Except the samples from the Egyptian vulture with ring 9070115, the rest of the analysed blood samples were preserved in heparin that can inhibit PCR processes, and dry swabs remained refrigerated / frozen for a considerable time (1 or 2 years), which could interfere with the results.

The results are summarized as follows:

- All individuals resulted negative for the detection of:
  - ***Avian mycoplasma*** in blood, oropharynx and ocular samples
  - ***Chlamydia*** in blood and oropharynx

- o ***West Nile*** in oropharynx
- o ***Bordetella*** in oropharynx

Avian Adenovirus:

- As explained in the results section for the groups **BG01, BG02, BG03, BG05 and BG06 (all samples taken in Bulgaria, presented at map 1) positive results were obtained, in all cases with low and extremely low positive results.**
- **The low virus concentration detected only means that these individuals had only contact with this virus, but were not affected – were not sick.**

Map 1: Positive samples groups/pools on Avian adenovirus

From the map we can see that all the low or extremely low positive results were from samples taken from the Bulgarian Egyptian Vultures population. From a previous research done by BSPB/HOS/WWF-Greece trough this Life project: "The Return of the Neohpron" we know that the birds form these two "geographically separate" sub-populations (the Ester and the South population) are using different wintering sites in Africa, but using very similar migratory route. Therefore, we suspect that there is possibility that contact with Avian adenovirus might have been established along the migration and not in the wintering or breeding sites. This of course cannot be concluded; it is only a possibility or starting theory for some further research.

The impact of this virus to birds can be high, as shown for example in work done in Falconiformes, where occurrence of 35% was detected in some species (26, 7).

Adenovirus infections are common in poultry, and probably in all avian species. There are wide ranges of virulence in some cases even within the same serotype. While many infections are subclinical (has been isolated from healthy birds) and minor, sometimes also it may be associated with disease outbreaks. In general not relevant to public health (not a zoonosis) (29). Vertical transmission is the most common route of infection. Born chickens can excrete the virus through faeces until 2-4 weeks of age. After the period of excretion, the virus remains in a latent state, presumably due to the development of local immunity.

The horizontal transmission is also important. The virus is excreted through the faeces and can also develop in the nasal and tracheal mucosa, conjunctiva and kidneys.

About the disease that produces: adenoviruses have been associated with respiratory diseases, diarrhea, failure of egg laying, loss of appetite and even arthritis. Most times the adenovirus acts as secondary rather than primary pathogen.

The primary lesion that is associated with adenovirus is the inclusion body hepatitis (29).

Avian Circovirus:

- As explained in the results section for the groups **BG01, BG04, BG05, BG07, GR01, GR02, GR03 (samples from Bulgaria and Greece, presented at map 2) positive results were obtained, in all cases with low and extremely low positive results.**

Map 2: Positive samples groups/pools on Avian circovirus

From this map we can see that the low or extremely low positive results are taken from Bulgaria and Greece (different sub-populations), population or sub-population that are using different breeding and wintering

sites. The only negative results where obtained of samples taken from the Ester Bulgarian population, something that makes it difficult to set a theory or possibility for possible location of the contact with the Avian circovirus. Anyways, we can only say that this could be a common virus widely dispersed.

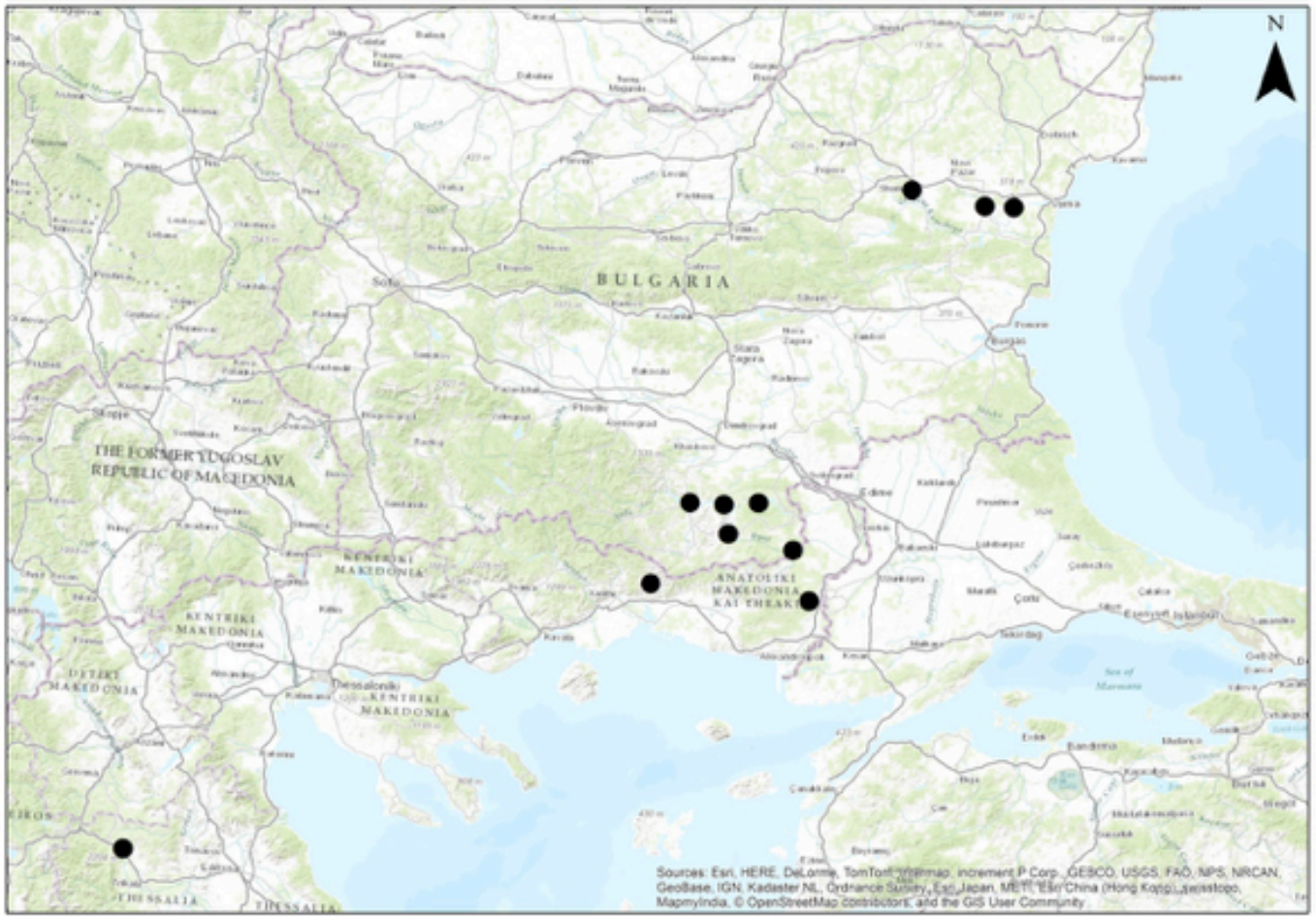
This virus is causing beak and feathers disease that can result with loss of feathers and beak malformation. The virus attacks the growing cells of follicles of the feathers, beak and claws causing progressive malformation and necrosis of the feathers. In advanced stages of the disease, the feathers develop constrictions in the spine, early ceasing its development until it eventually ceases all feather growth. The beak and nails are affected in the opposite direction: overgrowth, malformation and development of necrotic tissue. The cracking and peeling of the outer layers allow fungal infections to occur and further complications. Necrosis of the inner layers of the peak may cause its rupture, in which case the bird will be unable to feed. The disease also has a general immunosuppressive effect to the bird, opening the way to secondary viral and bacterial infection, which is often the cause of death and not the virus itself (27).

In this study we have obtained very low positive and extremely low, in absence of clinical symptoms in the analysed individuals, be consider this as an incidental finding not associated with disease in these individuals.

Newcastle:

- As explained in the results section in the Egyptian Vulture with **ring 9070115 samples, low positive results were detected in blood and oropharynx swab.**
- In the groups **BG01, BG03, BG04, BG05, BG06, BG07, GR01, GR02, GR03 positive results were obtained (presented at map 3), in all cases with low and extremely low positive results.**

Map 3: Positive samples groups/pools on Newcastle



Samples taken from all Bulgarian and Greek populations are presenting low or extremely low positive results on Newcastle, what brings to a conclusion that this is a widely dispersed virus in the breeding and the wintering areas.

This virus belongs to the group of paramyxovirus and has been isolated from avian species (domestic and wild birds), in some cases is associated with serious neurological problems and can cause death to the affected individuals (25, 28).

The results are considered extremely low, in the absence of neurological symptoms must be considered an incidental finding and not associated with disease in these individuals.

5.4. Pesticides analyses in blood

- **All individuals resulted negative to pesticides detection analyses in blood samples.**
- With these results we can confirm that the individuals sampled had no presence of these compounds in blood (last point in section 2 to be considered).

5.5. Antibiotics analyses in blood

- **All individuals resulted negative to antibiotics detection analyses in blood samples.**
- With these results we can confirm that the individuals sampled had no presence of these compounds in blood (last point in section 2 to be considered).

5.6. Heavy metals analyses: lead, zinc and cadmium

- It was insufficient sample material for analysing the three different metals. Since zinc is an element that accumulates very quickly in kidney, it is not usually detected in blood, preference was given to the analysis of lead and cadmium.
- **We have obtained a positive lead result from the blood sample of the Egyptian Vulture with ring 9070115, with a concentration of 22.88 µg/dL, indicating on recent exposure** (15, 16, 17, 18, 19, 20, 21). **This Egyptian Vulture was sampled in AMUS, afterwards was demonstrated that the bird was contaminated during its stay in the AMUS facilities.**
- In none of the analysed blood samples were quantified levels of cadmium. In the two of the groups (BG01 and BG03) was not possible to analyse these metals, as the sample quantity was insufficient.

5.7. NSAIDs analyses in blood

- The only positive result was obtained from the pool of blood samples from one of the groups analysed: GR02 (Greece)
- **Aspirin (acetylsalicylic acid) was detected with a concentration of 0.067 mg/kg.**
- The colleagues from the Hellenic Ornithological Society have identified that in the areas where these samples were taken (Greece), the Aspirin (acetylsalicylic acid) use is very common for treatment of turkeys and chickens.

The acetylsalicylic acid is a nonsteroidal antiinflammatory drug (NSAID) of common use in human practice (aspirin) and does not require a medical prescription. Its veterinary use is authorized in Spain (Spanish Medicines Agency; http://www.aemps.gob.es/). It is used in pets and domestic animals in general (as single compound or as part of other formulations): to promote wound healing, use in infected wounds after surgery; symptomatic treatment of pyrexia; in acute respiratory disease; or as an anti-inflammatory. Its common use also makes it one of the compounds that are detected more frequently in the waters of rivers throughout Spain (1, 2).

Regarding the toxicity of acetylsalicylic acid, it is classified with harmful ecotoxicological value (1,3,4,5). However the ecotoxicological risk on aquatic organisms is considered low after treatment of wastewater (6). After the literature review no data on toxicity to wild birds was found, although it has previously been detected in Griffon Vultures, Egyptian Vultures and owls before the crisis of Bovine Spongiform Encephalopathy (7).

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ANNEX: Recommendations for taking and conservation of biological samples for future analysis in the center of analysis and diagnosis of wildlife (CAD)

A) IMPORTANCE OF THE QUALITY OF THE SAMPLES AND MEDICAL HISTORY

The most important for the laboratory analysis is the sample. From a well-selected sample in sufficient quantity, well preserved and send in a correct time to the lab, all information related to the health of the animal (clinical condition, pathological and toxicology) can be obtained.

B) GENERAL RULES

- Sampling:

- The staff responsible for sampling must use (be provided with) personal protective equipment (PPE) during the sampling process (at least gloves and masks, disposable overalls is also recommended.).
- It is essential to collect aseptic samples especially for microbiological analysis due to possible interfer ence with the isolates. Avoid contact between the soil and the samples.
- Samples before sending must be individually identified (labelling, seal, sticker, etc.) clearly indicating the material (tissue, organ, etc.) containing, animal species and sampling date.

- Conservation:

- Frizzing (not later then 3 days after the sampling): blood in EDTA (to study various microorganisms by molecular diagnosis PCR), clean plasma and serums (after centrifugation of the blood samples). Swabs in virus medium.
- Refrigeration not more then 24-48 hours until the shipment:
  - 24 hours: blood in EDTA and heparin if it's for haematological or biochemical analysis.
  - 24-48 hours: swabs in conservation medium for microbiology (Amies) and virology (specific medium for virus).
  - 24-48 hours: faeces.

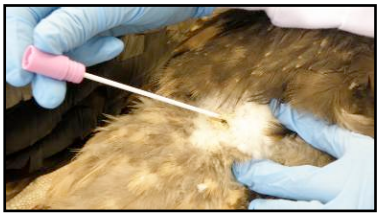
C) SAMPLING PROCEDURES

1. Collection of samples: SWABS FROM CLOCA IN MEDIUM FOR TRANSPORTATION AMIS ANS VIRUS MEDIUM

- Use to collect cloacal and / or oropharynx samples. Insert and turn the swab in rubbing effort to obtain cells.
- Number of swabs: ideally 1 per organism for study
- Analysis:
  - o Conservation in Amies medium (blue swab):
    - Microbiology: divers bacteria species: Salmonella, Campylobacter, Escherichia coli O157.

  - o Conservation in virus medium (pink swab):
    - Virology (molecular diagnostics PCR, virus cultivation). Different viruses.

Samples collection from cloaca with swab in Amies medium (blue)



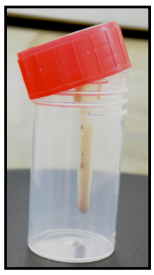
Samples collection from cloaca with swab in virus medium (pink)

Samples collection from oropharynx with swab in virus medium (pink)

- Also check the table at the end of this document.

2. Sample collection: FAECES

- Collect fresh faeces. Discard the urea phase.
- Use a sealed, sterile container – refrigeration.
- Possible analysis:
  - o Parasitology.
  - o Microbiology: Microbiology: divers bacteria species: Salmonella, Campylobacter, Escherichia coli O157.



3. Sample collection: BLOOD IN EDTA AND HEPARIN

- Take the sample by venepuncture, previously disinfect the area with 70% alcohol and wait at least 30 seconds.
- Fill in the sample tube with blood. Overturn smoothly 3-4 times for mixing the blood and the EDTA / heparin, to prevent lyse of the erythrocytes.
- Recommended volume: 1 tube of 1 ml EDTA to study molecular diagnostics (PCR); 1 tube EDTA for metals; 1 tube EDTA for haematology y sex determination; 1 tube heparin for biochemistry and proteinogram.
- Analysis from EDTA:
  - o Sex determination
  - o Molecular diagnostics (PCR): different microorganisms (bacteria, virus, parasites).
  - o Metals: lead, cadmium.
- Analysis from heparin: In this case is recommended to centrifuge the tube and recover the blood

plasma. This plasma can be refrigerated if the shipment is carried out immediately, or frozen.

- o Biochemistry
- o Proteinogram (serum protein profile)

4. Sample collection: SERUM

- Take blood into a tube for serum.
- To facilitate clot retraction leave reverse or leaning tube at room temperature about 30 minutes. If possible centrifuge (2500 rpm 10 minutes), and collect the clean serum in other tube aside. It can be freeze until its shipment to CAD.

- Recommended volume: 0,2-0,5 ml of clean serum for each analysis.
- Analysis: detection of antibodies against different microorganisms.

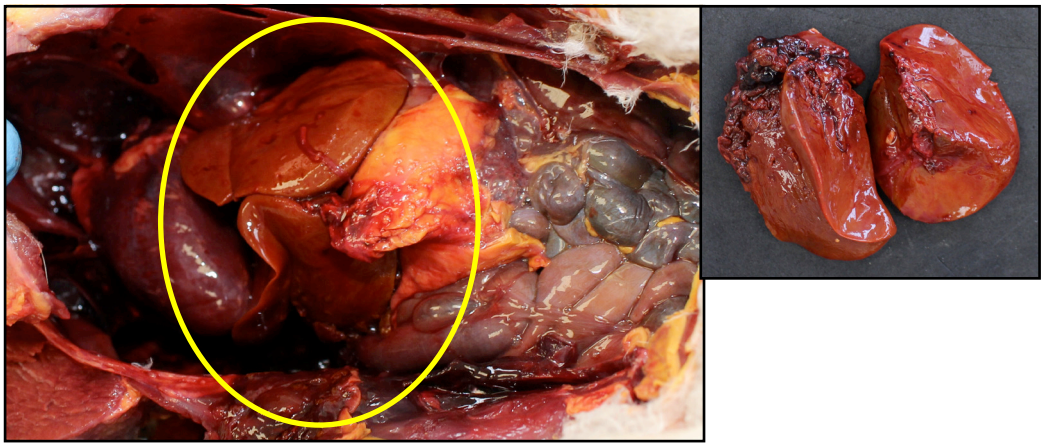
Decanting and passage of the clean serum in new tube (eppendorfs or other containers can be used for maintaining the serum, for example vials)

5. Sample collection: LIVER, KIDNEY, BONE, FEATHERS

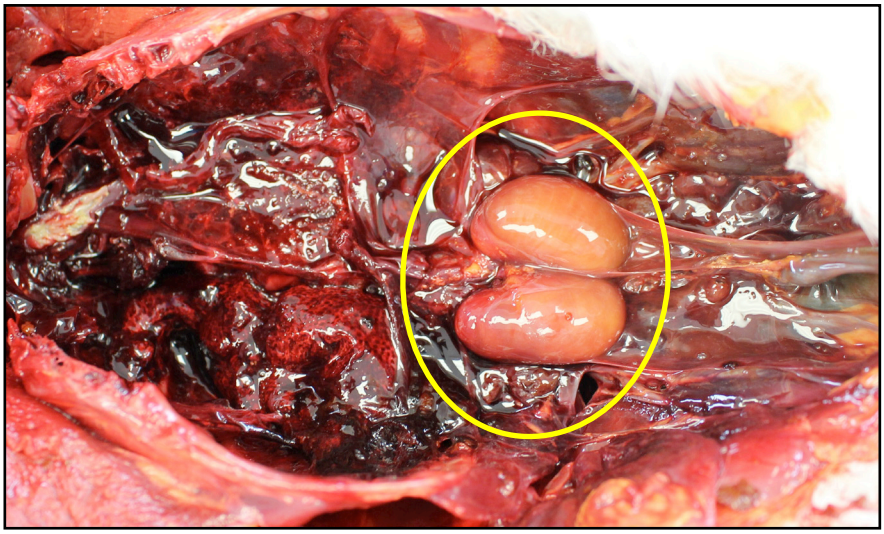
- After the necropsy take the complete liver and kidneys.
- Store in a clean container (preferably “flacon” or similar to secure the closure)
- Bone: select bone to obtain at least 10 grams. Emit in zip bag or sealed container.

- **Feathers: select primary or secondary feathers. If possible select feathers that are in growing and those that do not are. It is very important to note whether they are growing or not for proper interpretation of results (Recent poisoning or environmental contamination). About 10 grams in total are needed. Store in paper or plastic seal able bags.**

- **Analysis (depending of the sample, check the table below):**
  - **o Metals (lead, zinc, cadmium)**
  - **o Nonsteroidal anti-inflammatory NSAIDs**
  - **o Antimicrobials**
  - **o Pesticides**



Liver



Kidneys

Sampling procedure, C section	Sample	Conservation medium	Analysis	Conservation by refrigeration (4ºC)	Conservation by frizzing (-20ºC; -80ºC)	Comments
1	Swab	Amies	Microbiological	24-48 hours	No	
1	Swab	Virus	Virus study (PCR/ cell cultivation)	24-48 hours	-80ºC (recommendable)	Mayor congelation tº can interfere with the viability of some viruses (e.g. Influenza)
2	Faeces	No medium	Parasitological	24-48 hours	In comments	Freezing is not recommended, but some parasites and bacteria are viable after freezing on -20ºC to -80ºC
			Microbiological			
3	Blood	EDTA	PCR	Until 3 days	Yes	
			Metals (lead, cadmium)	Until 3 days	Yes	
			Sex (PCR)	Until 3 days	Yes	
			Lead, antibiotics, NSAIDs	24-48 hours	Yes	
		Heparin	Proteinigram	24 hours	No	The plasma can be frozen after centrifugation. Should not be hemolysate
			Biochemical	24 hours	No	
4	Serum	No medium	Immunological (antibodies)	Until 3 days	Yes	Is recommended to freeze if not immediate shipment
5	Feather	Paper envelope/no medium	Lead, cadmium	24-48 hours	Yes	Important to note whether it is growing or not (toxic accumulation of short and long term). Also applied the comments made for