Veterinary forensic sciences to solve a fatal case of predation on flamingos (Phoenicopterus roseus)

Silva Rubini[1*], Stefania Barbieri[2], Rosa Maria Gaudio[3], Guido Govoni[4], Giulia Rasera Berna[5], Rosario Fico[6], Rita Lorenzini[7], Maria Cristina Fontana[8], Roberta Taddei[8], Massimo Tassinari[9], Paolo Frisoni[3], Enrica Guidi[10] and Mauro Bergamini[10]

[1] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) Sezione di Ferrara, Via Modena 483, 44124 Cassana Ferrara, Italy.*
[2] *Azienda Ospedaliera Padova, Via Giustiniani 2, 35128 Padova, Italy.*
[3] *Università di Ferrara Dipartimento di Scienze Mediche c/o Sezione di Medicina Legale e delle Assicurazioni, Via Fossato di Mortara 64, 44121 Ferrara, Italy.*
[4] *Economic Security Unit Head of Sector Horn of Africa/Veterinarian, International Committee of the Red Cross (ICRC), 19 Av. de la Paix 1202 Genève, Switzerland.*
[5] *Biologist freelancer, Via Medaglie d'oro 33, 32100 Belluno, Italy.*
[6] *Laboratorio Nazionale di Riferimento per la Medicina Forense Veterinaria, Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Sezione di Grosseto, Viale Europa, 30, 58100 Grosseto, Italy.*
[7] *Laboratorio Nazionale di Riferimento per la Medicina Forense Veterinaria, Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Sezione di Rieti, Via Tancia, 21, 02100 Rieti, Italy.*
[8] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione di Bologna, Via P. Fiorini 5, 40127 Bologna, Italy.*
[9] *Azienda usl Ferrara - Dipartimento di Sanità Pubblica, M.O. Sanità animale, Via Cassoli 30, 44121 Ferrara, Italy.*
[10] *Sezione di Medicina di Sanità Pubblica, Dipartimento di Scienze Mediche, Università di Ferrara, via Fossato di Mortara 64, 44121 Ferrara, Italy.*

[*] *Corresponding author at: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione di Ferrara, Via Modena 483, 44124 Cassana Ferrara, Italy. Tel.: +39 0532 730058, Fax: +39 0532 730870, e-mail: silva.rubini@izsler.it .*

Veterinaria Italiana 2018, **54** (2), 175-180. doi: 10.12834/VetIt.1360.7488.1
Accepted: 01.08.2017 | Available on line: 30.06.2018

Keywords
Dog,
Flaming,
Forensic,
Fox.

Summary
The present case study concerns a case of predation of 4 individuals of captive pink flamingo in Emilia Romagna Region, Northeastern Italy. The pink flamingo (*Phoenicopterus roseus*) is a species included in the Red List of Threatened Species established by the International Union for Conservation of Nature (IUCN) which lists species in danger of extinction. During the Winter of 2013, 4 flamingos (2 in the Comacchio area, and 2 from Argenta and Codigoro oases – Ferrara province) were found dead some of them headless, with their bodies severely bitten. At first, a fox (*Vulpes vulpes*) was suspected to be the predator responsible for the killing and the birds were taken to the laboratory for further investigations. The investigations included: field observations, study of the predator behaviour, necropsy examinations, assessment of the intercanine distance, and genetic analysis on the predator's traces. The intercanine distance indicated that the predator could not have been a fox. The analysis of salivary DNA samples enabled us to establish that the predator was in fact a dog. This case highlights the importance of co-operation among the various branches of forensic sciences and the great usefulness of the roles filled by other veterinary forensic experts involved in solving crime.

Un caso fatale di predazione sui fenicotteri (Phoenicopterus roseus) risolto con la scienza veterinaria forense

Parole chiave
Cane,
Fenicottero,
Scienza forense,
Volpe.

Riassunto
In questo studio si presenta il caso di 4 fenicotteri trovati morti, nell'inverno del 2013, con i corpi gravemente morsi e alcuni dei quali senza testa, in un'oasi di conservazione per la riabilitazione della fauna selvatica nella quale erano stati condotti perché incapaci a volare. Gli uccelli sono stati portati in laboratorio per ulteriori indagini sospettando, in prima ipotesi,

che una volpe (*Vulpes vulpes*) fosse responsabile dell'uccisione. Le indagini hanno incluso: osservazioni sul campo, studio del comportamento dei predatori, esami di necropsia, valutazione della distanza intercanina e analisi genetiche sulle tracce del predatore. La distanza intercanina ha evidenziato che il predatore non potesse essere una volpe e l'analisi dei campioni di DNA salivare ha permesso di stabilire che il responsabile fosse in realtà un cane. Questo caso sottolinea l'importanza della cooperazione tra i vari rami delle scienze forensi e la grande utilità dei ruoli ricoperti da altri esperti di medicina legale coinvolti nella risoluzione del caso.

Molecular methods have been applied in various fields of biology, including Medical/Health Science. In this paper, we use DNA analysis in the veterinary field in order to identify the species linked to a form of predation. Although the DNA analysis clarifies important aspects of veterinary medicine, it does not comprehensively address clinical questions, especially in the field of forensic veterinary medicine. Defining the causes of death of an animal subject to predation involves the evaluation of multidisciplinary aspects. The role of DNA analysis in this process is to confirm what emerged from the examination of the crime scene, the necropsy evaluation, and analyse the morphological findings connected to the predator. This study presents a case in which the animal species responsible for a case of predation against a group of pink flamingos is identified. The pink flamingo (Phoenicopterus roseus) is a species included in the Red List of Threatened Species established by the International Union for Conservation of Nature (IUCN), which lists species in danger of extinction. Thanks to the protection guaranteed by zoos as well as the establishment of protected areas, the number of specimens of pink flamingos is currently on the rise (BirdLife International 2017). In Italy, this species had initially been spotted in Sardinia. It later appeared in mainland areas such as Tuscany, Puglia, and the Northwestern Adriatic area, where it has been regularly reproducing in Comacchio, Ferrara province, since 2000 (Costa *et al.* 2009, Sanz-Aguilar *et al.* 2012). In natural environments, the predation of healthy adults is very rare. The predation of unhealthy animals or animals that are unable to fly in a man-protected environment can be considered exceptional (Bechet & Johnoson 2008, Tourenq *et al.* 2001). When this rare circumstance does occur, it is therefore necessary to investigate. The present case study concerns a case of predation of 4 individuals of captive pink flamingo that, unable to fly, were kept by a local centre for rehabilitation of wildlife. The centre was managed by LIPU (Lega Italiana Protezione Uccelli – Italian League for Bird Protection), an Italian partner of Birdlife International. In this centre, wounded and sick animals receive veterinary treatment before their release into the wild, otherwise they are kept under suitable captivity conditions for biological and environmental teaching purposes. The present study aims to identify the predator through analysis of its behavior, pathological investigation, and DNA analysis.

Our pink flamingo sample set consisted of 2 adults found in the Comacchio area with broken wings due to shooting, and 2 young birds from Argenta and Codigoro oases. All the birds were found dead, some of them headless, although the bodies were still well preserved. A fox (*Vulpes vulpes*) was initially suspected of the killings. The birds were taken for further investigations to Ferrara diagnostic Centre, a satellite laboratory of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), where they were registered and a pathology examination took place. Before the necropsy, the carcasses were refrigerated at 4 °C. Research relating to the intercanine distance (Tedeschi-Oliveira *et al.* 2011) as determined by the lesions was carried out. Swabs from the edge of the lesions, identifiable as bites, were collected in order to analyze the predator's saliva (Reedy *et al.* 2011). Particular attention was taken to avoid contamination with the blood of the birds. In total, 6 swabs were collected as follows: 2 from flamingo 1, 1 from flamingo 2, 1 from flamingo 3, and 2 from flamingo 4. All the swabs were protected in appropriate plastic containers, frozen, and checked for predator DNA. Analysis was carried out at the National Reference Centre for Forensic Veterinary Medicine (Centro di Referenza Nazionale per la Medicina Forense Veterinaria), in the Rieti laboratory, a diagnostic Centre of the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana. The DNA was extracted using the DNA IQTM Casework Sample Kit and the semiautomatic extractor Maxwell 16 LEV (Promega). Since salivary DNA is often damaged (Williams *et al.* 2015) and in small quantities, a limited portion – 188 base pairs long – of the ND1 mitochondrial gene were amplified (Kenna *et al.* 2011). The high number of copies of mitochondrial DNA per cell increased the chances of a successful amplification via polymerase chain reaction (PCR). The primer pairs, selected by the National Reference Centre for Forensic Veterinary

Medicine, amplify the DNA of animals that specifically belong to the *Canidae* family. This method selects and sequences the predator's DNA only. In each session, an extraction and amplification control set was included. Once amplified, the segment was sequenced in both directions by using the same primers in conjunction with the BigDye Terminator v.3.1 kit (Applied Biosystems) through the automatic sequentiator Genetic Analyzer 3130 (Applied Biosystems). In addition to ND1 mitochondrial gene sequencing, an analysis of microsatellites, or short tandem repeats (STR) was carried out. A panel of 20 microsatellites loci was analysed; the loci were co-amplified in 5 different multiplex PCR reactions, according to the method described by Caniglia and colleagues and Lorenzini and colleagues (Caniglia *et al.* 2013, Lorenzini *et al.* 2014).

The first inspection of the site was carried out when the dead birds were discovered (14 January 2013). A more accurate inspection was repeated 2 days later in order to gather details that would be useful in identifying the predator.

Analysis of the crime scene revealed that only a few parts of the birds had been eaten (Figure 1). The carcasses were almost equidistant from each other and lying at the edge of the artificial lake situated inside the fence. Three of the 4 flamingos had been beheaded. The head of 1 flamingo lay at a distance of about 45 cm-50 cm from its body. The heads of the other 2 beheaded subjects have never been found. All of these aspects of the crime scene suggest the behavior expression of a fox (Fleming *et al.* 2016, Moberly *et al.* 2004). Indeed foxes usually remove parts of the body, mainly the head, in order to eat them later on (Kaczensky *et al.* 1998).

Local investigation also included the analysis of specific footprints (Krishan 2008) in order to help identify the species responsible for the killing. Some prints were found; although they were not clear enough to identify any particular species. The first inspection of the area did not reveal breakages in the protective fence, reinforcing the hypothesis that a predator had to climb the fence to reach the flamingos. Two days later, when it became possible to examine the entire fence more thoroughly, a gap through which a predator could have entered was identified. The gap was 30 cm long and about 15 cm width in the centre. Unfortunately, it was too late to collect evidence such as hair that may have been caught in the net.

The necropsy was carried out 36 ± 3 hours post mortem. The carcasses of the 4 flamingos had been checked carefully for external traumatic lesions. The birds were consequently skinned in order to gain a better understanding of the lesions and then each apparatus was deeply examined. The exam highlighted superficial, deep lesions and signs of bites, which were recorded through the measurement of intercanine teeth distance (Figure 2). The plumage of all 4 subjects was smeared with mud in where it had been exposed to the external environment. By contrast, the areas located under the wings were clean. In the days before the flamingos' death, it rained heavily and the ground of the fence got wet. Next to each body only a few feathers were torn or detached. In all subjects, the proventriculus was empty, and the gizzard contained feed and grit (Figure 3).

Sample 1 was an adult male. The head and neck were severed with lacerations of the soft tissues and rupture at the base of the cervical spine. The soft tissues located at the extremities of the lacerations, both on the neck and on the body, were soaked in blood and the margins of the wounds appeared swollen and bleeding. Even the feathers surrounding these lesions were bloody. This evidence shows that the injuries were inflicted when the animal was still alive. About 10 cm of the distal portion of the tracheal tube had been cleanly removed. The stump of the neck had jagged edges and several vertebrae were fractured and injured. The head of this flamingo, found 45 cm-50 cm away from the

Figure 1. *All birds were found dead, some of them headless.*

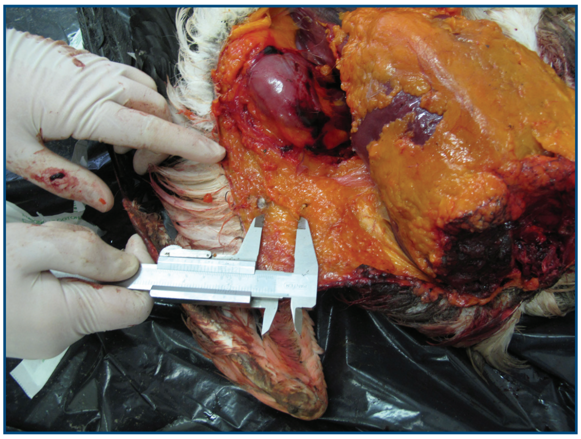

Figure 2. *Measurement of the intercanine distance of the predator.*

Figure 3. *Necropsy findings.* **A**. Flamingo n. 1. **B**. Flamingo n. 2. **C**. Flamingo n. 3. **D**. Flamingo n. 4.

body, did not show injury: beak, eyes, and feathers were unharmed. The wings and feet did not show any lesions. The body wall was intact. The coelomic cavity as well as the internal organs did not present any macroscopic lesions.

Sample 2 was an adult of undetermined sex. It was not possible to determine the sex of this flamingo during the necropsy, however, dimensions and anamnestic information indicate it was a female. This was the only flamingo that did not have its head and neck severed. The feathers of the ventral region were heavily soiled with mud and soaked in water; the animal was grounded and remained in this position until the carcass was discovered the morning after its death. The left side of the body had a laceration almost parallel to the distal edge of the sternum, approximately 6 cm long and approximately 3 cm wide. Part of the thoracolumbar spine had been removed. Numerous coagulations were visible in the coelomic cavity. There were severe bruises on the left thigh. The wings and feet did not show any lesion. The kidneys, genitals, organs, and most of the lungs were excised.

Sample 3 was a juvenile female. This subject was found without its head and neck. At the entrance of the chest there was a lacerated and bruised wound surrounded by bleeding and swollen soft tissue. Both feathers of the ventral region of the body were smeared in what appeared to be an abundant blood loss. Bite marks were observed on the left thigh, near the knee. The clearest hole had an oval shape (8.0 mm x 5.0 mm), and a depth of 8.0 mm (to the bone). After skinning, we observed numerous bruises on the left thigh. In the coelomic cavity there was abundance of blood. The left lung was removed and the liver appeared torn. This animal also lacked a portion of the small intestine. The wings and the feet did not show any lesion.

Sample 4 was a juvenile female. This subject was also found without its head and neck. The ventral region was smeared with blood. On the right thigh there was an evident bite mark. After skinning, lesions appeared oval in shape (8.0 mm x 4.0 mm), and 8.0 mm deep (to the bone). Bleeding and subcutaneous and muscular tissue reaction confirmed that the animal was alive at the time of the attack. The intercanine distance of 20.0 mm was measured with a caliper in the subcutaneous tissue of this subject. The sampling area was located on the abdominal wall, under the sternum. In this sample, the wings and the feet did not show any lesions.

The predator's DNA was successfully extracted from each flamingo. Amplification and sequencing of a portion of the mitochondrial ND1 gene showed, through comparison with control sequences and international databases (GenBank), that salivary traces on the wounds belonged to the *Canis lupus* species and not to *Vulpes vulpes*, as initially suspected. Mitochondrial DNA analysis does not, however, reveal a distinction between wolf and dog (both belonging to the *C. lupus* species). An analysis of nuclear DNA through the amplification of 20 STR loci was therefore required. This analysis showed that the salivary DNA belonged to a dog, further excluding the possibility that a wolf was the predator. Unfortunately, since only 1 extracted DNA sample provided a nucleotide sequence fitted for the analysis, it was not possible to determine how many predator there were.

To our knowledge there are no studies describing cases of attacks on flamingos by dogs, because flamingos and dogs normally live in different environments. Therefore, the attack we describe in this study is considered objectively rare and improbable. Flamingos frequently stand on 1 leg, primarily to maintain body temperature and reduce the loss of heat during cold periods. The position is most likely very comfortable for them as it is used during the hottest periods as well as during times of rest (Grzimek and Eibl-Eibesfeldt 2002). This was most likely the position of the animals on the night of the attack, which increased their vulnerability.

In this study a site inspection was carried out, but a reconstruction of the crime scene was not possible due to adverse climatic conditions. At first a fox was suspected to be the predator. The technical confirmation that a fox predation would be excluded came from the measurement of the intercanine distance. The intercanine distance is a species-specific element and is therefore useful in identifying predators (Ratz *et al.* 1999). With particular reference to an adult fox, the average intercanine distance is 24 mm-27 mm (Hart 1982), that is greater than those that we documented through flamingos necropsy. We were able to hypothesize that the predator could be a dog (*Canis lupus familiaris*). In dogs there is high variability in intercanine distance in relation to the breed and size of the individuals. Among small dogs, typical intercanine distances of 29 mm in the upper jaw, and 25.6 mm in the lower jaw may be observed. In spite of this, lesser values such as 22 mm for the upper jaw and 18 mm for the lower jaw, respectively (Tedeschi-Oliveira *et al.* 2011), have been recorded for very small sized dogs. This is in-line with the findings of the present case. Unfortunately, the detection of intercanine distances could only be carried out in 1 of the specimens. The extension of the lacerations observed in the other 3 samples prevented us from accurately measuring the intercanine distance. Because of this, a biomolecular research was carried out to further confirm the predator. Swabs from the edges of the lesions (identifiable as bites) were collected to check the predator's saliva. Sequencing a portion in the mitochondrial ND1 gene showed salivary traces on the wounds that related to the *Canis lupus* species. The STR analysis further identified the predator as a dog who had probably escaped surveillance during a walk in the nearby park.

Our study demonstrates that the molecular identification of DNA can be successfully used during forensic investigations in order to identify unknown predators. In cases when the reconstruction of the crime scene and/or the conservation of the dead animals are compromised, molecular analysis can in particular be advantageous. Integrated studies of veterinary and forensic sciences have the potential to yield important details about the circumstances and dynamics of a predator attack, and molecular biology can confirm any hypothesis that are put forward. This case highlights the importance of co-operation among the various branches of the forensic sciences and veterinary forensic experts (Byard and Boardman 2011, Cooper and Cooper 2008). Although forensic pathologists have a central role in determining the reconstruction of an accident, this branch of forensics is not currently used in daily veterinary medicine (Aquila *et al.* 2014). A European international survey of pathology laboratories and institutes reported a scarcity of training opportunities and special education, and insufficient veterinary-specific reference data and information on forensic analyses (Ottinger *et al.* 2014). A recent study observed that almost 75% of veterinary pathologists report that their training has not adequately prepared them to handle forensic cases (McDonough and McEwen 2016). As is reported in other studies (Cooper *et al.* 2009, Ottinger *et al.* 2014), the present study underlines the importance of the veterinary forensic approach in investigating the diagnostic forensic autopsy of flamingo deaths. Our study is able to demonstrate correlations between the crime scene, location, DNA results, and circumstances of a lethal attack, and our findings were an integral part of this investigation.

This study introduces an integrated and multidisciplinary approach to identify the predator responsible for an attack, and thus stands to contribute to the future practice of veterinary forensic pathology.

Acknowledgements

We would thank Lorenzo Borghi, Davide Tartari (LIPU – Lega Italiana Protezione Uccelli – Sezione di Ferrara) for their kind cooperation and precious contribution in sampling activities.

References

Aquila I., Di Nunzio C., Paciello O., Britti D., Pepe F., De Luca E. & Ricci P. 2014. An unusual pedestrian road trauma: from forensic pathology to forensic veterinary medicine. *Forensic Sci Int*, **234**, 1-4.

Bechet A. & Johnson AR. 2008. Anthropogenic and environmental determinants of greater flamingo (*Phoenicopterus roseus*) breeding numbers and productivity in the Camargue (Rhone delta, southern France). *Ibis*,**150**, 69-79.

BirdLife International. 2017. *Phoenicopterus roseus*. The IUCN Red List of Threatened Species 2017: e.T22697360A119273745. http://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T22697360A119273745.en.

Byard R.W. & Boardman W. 2011. The potential role of forensic pathologists in veterinary forensic medicine. *Forensic Sci Med Pathol*, **7**, 231-232.

Caniglia R., Fabbri E., Mastrogiuseppe L. & Randi E. 2013. Who is who? Identification of livestock predators using forensic genetic, approaches. *Forensic Sci Int, Genetics*, **7**, 397-404.

Cooper J.E. & Cooper M.E. 2008. Forensic veterinary medicine: a rapidly evolving discipline. *Forensic Sci Med Pathol*, **4**, 75-82.

Cooper J.E., Cooper M.E. & Budgen P. 2009. Wildlife crime scene investigation: techniques, tools and technology. *Endang Species Res*, **9**, 229-238.

Costa M., Baccetti N., Spadoni R. & Benelli G. 2009. Monitoraggio degli uccelli acquatici nidificanti nel Parco del Delta del Po Emilia-Romagna. Giari Ed., Ferrara.

Coyle H.M. 2012. The importance of scientific evaluation of biological evidence-data from eight years of case review. *Science & Justice*, **52**, 268-270.

Fleming P.A., Dundas S.J., Lau Y.Y. & Pluske J.R. 2016. Predation by red foxes (*Vulpes vulpes*) at an outdoor piggery. *Animals*, **6**, 1-17.

Grzimek's Animal Life Encyclopedia. 2002. 2nd Ed., Farmington Hills, Michigan.

Hart E.B. 1982. Intercanine crown distances in red foxes and badgers. *Great Basin Naturalist*, **42**, 601-602.

Kaczensky P., Huber T., Huber D., Frkovic A. & Fico R. 1998. Chi è stato? Riconoscere e documentare gli animali da preda e le loro tracce. Teramo, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise.

Kenna J., Smyth M., McKenna L., Dockery C. & McDermott S.D. 2011. The recovery and persistence of salivary DNA on human skin. *J Forensic Sci*, **56**, 170-175.

Krishan K. 2008. Establishing correlation of footprints with body weight-forensic aspects. *Forensic Sci Int*, **179**, 63-69.

Lorenzini R., Fanelli R., Grifoni G., Scholl F. & Fico R. 2014. Wolf-dog crossbreeding: "Smelling" a hybrid may not be easy. *Mammalian Biology*, **79**, 149-156.

McDonough S.P. & McEwen B.J. 2016. Veterinary forensic pathology: the search for truth. *Vet Pathol*, **53**, 875-877.

Moberly R.L., White P.C. & Harris S. 2004. Mortality due to fox predation in free-range poultry flocks in Britain. *Vet Rec*,**155**, 48-52.

Ottinger T., Rasmusson B., Segerstad C.H., Merck M., Goot FV., Olsén L. & Gavier-Widén D. 2014. Forensic veterinary pathology, today's situation and perspectives. *Vet Rec*, **175**, 459.

Ratz H., Moller H. & Fletcher D. 1999. Predator identification from bite marks on penguin and albatross chicks. *Marine Ornithology*, **27**, 149-156.

Reedy C.R., Hagan K.A. & Marchiarullo DJ. 2011. A modular microfluidic system for deoxyribonucleic acid identification by short tandem repeat analysis. *Anal Chim Acta*, **687**, 150-158.

Sanz-Aguilar A., Béchet A., Germain C., Johnson A. & Pradel R. 2012. To leave or not to leave: survival trade-offs between different migratory strategies in the greater flamingo. *J Anim Ecol*, **81**, 1171-1182.

Tedeschi-Oliveira S.V., Trigueiro M., Oliveira R.N. & Melani RF. 2011. Intercanine distance in the analysis of bite marks: a comparison of human and domestic dog dental arches. *J Forensic Odontostomatol*, **29**, 30-36.

Tourenq C.S., Aulagnier L. & Durieux S. 2001. Identifying rice fields at risk from damage by the greater flamingo. *J Appl Ecol*, **38**, 170-179.

Williams S., Panacek E., Green W., Kanthaswamy S., Hopkins C. & Calloway C. 2015. Recovery of salivary DNA from the skin after showering. *Forensic Sci Med Pathol*, **11**, 29-34.