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ATSB RESEARCH AND ANALYSIS REPORT
Aviation Safety Research Grant – B2005/0117
Final

Forensic Identification of Aviation Bird Strikes in Australia

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Postal address: PO Box 967, Civic Square ACT 2608

Office location: 15 Mort Street, Canberra City, Australian Capital Territory

Telephone: 1800 621 372; from overseas + 61 2 6274 6590

Facsimile: 02 6274 6474; from overseas + 61 2 6274 6474

E-mail: atsbinfo@atsb.gov.au

Internet: www.atsb.gov.au

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Author(s)

Christidis, L., Norman, J.A., Johnson, R.N. and Lindsay, S.

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www.atsb.gov.au

Abstract

The aim of this study was to investigate the feasibility of forensic DNA-based techniques in identifying species involved in Australian aviation bird strikes. Experimental bird tissues were subjected to severely damaging conditions to determine if DNA could be extracted from these samples. In addition, DNA and feather microscopy databanks were created from the species classified as being the 'highest risk' for strikes to provide reference data to compare against unknown samples. Finally, a DNA sampling kit was created and distributed widely to aerodromes across Australia for collection of material from unknown strikes for DNA analysis. Results of experimental bird tissue experiments showed the most detrimental conditions for DNA were to leave a sample at room temperature for 7+ days. DNA was successfully extracted from all strike samples collected with sampling kits then returned to the laboratory and positive identifications were able to be made to species level in the majority of cases. Interestingly, it was found that attempts at visual species identification were often incorrect and that the putative 'high risk' species were only responsible for 27 per cent of the unidentified strikes. In general, we found DNA identification of strike species to be a reliable method for identifying the species involved in collisions and conclude that it would be a useful addition to the methods already employed to identify wildlife strikes at civilian aerodromes.

ACKNOWLEDGEMENTS

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Finally, the authors sincerely thank all participating aerodromes, particularly maintenance engineers, and environmental and safety coordinators. Without the contributions and assistance from personnel at these aerodromes this project would not have been possible.

ABBREVIATIONS

ATSB	Australian Transport Safety Bureau
Cyt *b*	Cytochome *b*
COI	Cytochrome Oxidase I
DNA	Deoxyribonucleic acid
kPa	Kilopascals
mtDNA	mitochondrial DNA
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
Psi	Pounds per square inch
RT	Room temperature
SEM	Scanning Electron Micrograph

EXECUTIVE SUMMARY

The primary aim of this project was to investigate the feasibility of using forensic DNA testing to identify wildlife strikes that until now have been unidentifiable due to lack of morphological characteristics. To do this, a multi-faceted approach using both DNA-based and high magnification microscopy techniques was employed.

Aircraft are exposed to extreme environments that may cause DNA to become degraded. DNA-based experiments were carried out to determine how badly a sample could be damaged before it was not able to be analysed.

Experimental bird tissues were exposed to severely damaging conditions. An attempt was then made to extract DNA from these samples and amplify two genes, which usually amplify readily under non-damaging conditions. The results from these experiments showed one of the treatments most damaging to DNA is to be left at room temperature for 7+ days. If DNA testing were to be routinely used to identify unknown strike samples, this result has implications for the procedures that should be followed during sample collection to ensure the greatest chance of a positive DNA result.

To assist with the identification of unknown samples, a set of reliable DNA and microscopy data from vouchered[1] museum specimens was generated.

The DNA and feather microscopy databank from the Australian Transport Safety Bureau's previously published list of highest risk strike species (top eight from this list). The basis for this was to provide a reference set of data against which to compare unknown strike samples. Interestingly, only 27 per cent of strike samples analysed during this project was identified as species from this list. This is not surprising given the general nature of the list, which covered all of Australia. It would be overly ambitious to expect these species to occur as the top eight species in all parts of a country as large and climatically varied as Australia (providing a large number of habitats). It may also be a little unrealistic to expect that all eight species would represent a high proportion of all strikes.

High magnification analysis of different contour feather sections showed morphological characteristics that appeared to be diagnostic to species. The only limitation for this technique however, is that expert knowledge for comparison of these morphological characteristics is required for definitive identification to species using these techniques.

A field applicable DNA sampling kit was developed and distributed to aerodromes all over Australia so that unknown strikes could be sampled and returned to the laboratory for DNA identification.

1 An individual animal, part of an animal, or lot of specimens held in a museum collection. Associated with any voucher specimen are collection data, which detail the taxonomy, date and locality of collection, habitat, the collector and other relevant curatorial details. Voucher specimens fulfil an archival role by permitting identification to be checked thus permitting verification if necessary. Further information and guidelines can be found at http://www.agric.nsw.gov.au/reader/wildlife-research/arrp-voucher-specimens.htm

Samples from wildlife strikes returned with the kits included muscle, intestine, skin and feathers, none of which were identifiable by morphological characteristics. On all occasions, DNA was extracted and the samples were successfully identified to a species (with the exception of one sample, which was accidentally contaminated before it reached the laboratory).

The most interesting trend suggested by the strike samples indicate that identifications based on animal parts by aerodrome staff may not always be accurate. Collectors were asked to provide a putative identification if possible on the collection slip (see appendix 3; figure 6). On the 14 occasions that putative identifications were provided, DNA analysis revealed 10 (or 71 per cent) to be incorrect. Based on the strike samples, which were predominantly ‘unknowns’, little weight can be given to this error rate. Certainly, it is more difficult to identify only part of an animal and collectors may have been more likely to record a strike as unknown to the ATSB rather than include a putative species (as requested on the collection sheet).

Aerodrome safety staff generally do an excellent job of managing safety, and identifying species involved in strikes is just one of their many responsibilities. However, if the actual error rate is even a fraction of the 71 per cent recorded in the sample, this has the possibility to seriously affect the management of problem species and run the risk of recording the incorrect species responsible for a strike.

The DNA method developed in this project has demonstrated its validity through its ability to identify previously unknown samples and also provide extremely accurate data on strike species.

1 INTRODUCTION

Bird and mammal (or wildlife) strikes are estimated to cost the airline industry many millions of dollars annually though unscheduled aircraft down-time and actual damage to aircraft (United States Department of Agriculture, 2000). In accordance with the *Transport Safety Investigation Act and Regulations 2003*[2], a collision with an animal, including a bird, is to be reported to the Australian Transport Safety Bureau (ATSB) within 72 hours of the strike. Those aircraft involved in air transport operations are required to submit a bird strike report, irrespective of where the strike occurred. However, for those aircraft involved in operations other than air transport, a report is only necessary when the strike occurs on a licensed aerodrome. Figures available from the ATSB indicate that the number of strikes reported to the ATSB is increasing over time (Australian Transport Safety Bureau, 2006). This trend of increasing strikes has also been observed in other countries where similar data is recorded, such as Canada, Europe, the United Kingdom, and the United States (Transport Canada, 2004). In addition, there is recent evidence to suggest that the patterns of some migratory birds are also changing in response to varying climatic conditions (Both, Bouwhuis, Lessells & Visser, 2006). This is of particular interest as migratory birds can be responsible for a large number of strikes at certain times of the year.

When a strike occurs, accurate species identification is essential so that airlines and aerodromes can more appropriately and effectively manage what are deemed to be 'high risk species'. Currently, identification is done visually by the pilot, crew, air traffic controllers, or from what remains of carcasses after a strike. Birds in particular can be very difficult to identify, even for ornithological specialists, either on the wing or if only a few feathers remain. Based on current data available from the ATSB, more than 50 per cent of strikes are currently not able to be identified to any level of zoological classification. Accurate identification of strike species is especially important because:

- Correctly identifying a species allows for an accurate estimate of the weight and size of any birds involved in the strike, providing engineers additional information in assessing the potential damage to an aircraft.
- If a species can be accurately identified, predictions can be made about whether it is a flocking or solitary species, allowing for estimates of the number of individuals.
- The occurrence of a bird strike appears to be increasing in Australia. Reducing the level of unknown species (currently >50 per cent) would enable aerodromes and airlines to better manage the real problem species revealed by a more complete dataset.
- It is important to monitor the different species involved in strikes and changes in species representation over time. This is particularly important with respect to migratory birds, which are frequently involved in strikes, particularly in Northern Australia.

2 Transport Safety Investigation Act 2003 Part 3 Section 19; Transport Safety Investigation Regulations 2003 Section 2.4.

One potential solution to identifying the currently ‘unknown’ strike data is to carry out DNA-based identification of these unknown samples. This method of identification usually requires only a small sample of blood or tissue and is routinely used in the forensic identification of trace samples of humans, plants insects and other wildlife.

The overarching aim of this project was to determine if DNA can be obtained reliably from wildlife strike samples, thereby offering a potential solution to identifying the current 50 per cent of strikes that are classified as ‘unknown’ species. To achieve this aim the following experiments were conducted:

1) DNA can be damaged by extreme environmental conditions such as high heat or chemical contamination; test tissues were subjected to several environmental conditions similar to those of commercial aircraft.

2) The ATSB has already identified several species as high risk for strikes. DNA sequences from vouchered reference tissues[3] for eight of these species were obtained so as to provide a reference DNA databank to compare against unknown samples.

3) A field applicable DNA sampling kit was developed, which was distributed to aerodromes for the sampling of strikes. Following this, techniques for DNA extraction and identification from strike samples were developed.

4) Vouchered reference feathers for high risk species were obtained to provide a reference feather databank to compare against unknown feather samples.

3 Reference tissues were taken from voucher specimens where possible. This ensured that these specimens had been identified by a specialist and therefore materials from these specimens could act as reference DNA or reference feathers.

2 METHODOLOGY

2.1 DNA experiments

Several DNA-based experiments were designed to examine the conditions that samples may encounter on an aircraft, establish a DNA databank of high risk species, and to establish a field applicable collection and extraction technique for strike samples.

2.1.1 Test tissues

The first question was could DNA be extracted from samples where the DNA was degenerated? Extreme conditions such as high temperature and carbonised contamination may be damaging to the DNA in tissue deposited on engine parts. The aim of these experiments was to determine if DNA of any quality and quantity could be extracted after being subjected to extreme conditions. Treatments were conducted on chicken liver pieces (~0.3 grams each). The three conditions we chose are listed below and were carried out in all combinations:

- Drying: centrifugation in a vacuum centrifuge for 3 hours
- Autoclaving: 1 hour at 121ºC, 15psi (103.5 kPa)
- Heating: 90ºC for ~3 hours
- Carbon: tissue rubbed on blackened carbonised residue of burnt petrol

Treatments were conducted in quadruplicate so that each set was replicated and half the samples could be exposed to carbon residue from petrol (for further detail see appendix 1). To test the effect of different storage conditions after treatment one of the replicates was stored at -80ºC (optimal storage condition) and the other replicate was stored at RT for 7 days (sub-optimal storage condition mimicking a forgotten sample).

Following treatment and storage DNA was extracted from the samples and fragments from two genes (mtDNA Cytochrome *b* and an intron from the nuclear gene GAPD: glyceraldehyde-3-phosphate dehydrogenase) were amplified. These genes were chosen because they are typically easy to amplify, therefore failure to amplify almost certainly indicates DNA damaged beyond amplification. Cytochrome *b* is a mitochondrial gene and GAPD is nuclear. Mitochondrial genes are typically present in higher copy number than nuclear genes and are usually better preserved by virtue of certain physical characteristics (eg mitochondrial DNA is circular, nuclear DNA is linear).

2.1.2 DNA from high risk species

A rapid isolation technique was developed for extracting DNA from samples collected with the DNA sampling kit. Commercial DNA extraction kits UltraPure (distributed by Geneworks for Mo Bio) and QIAamp micro (distributed by Qiagen) were used for this purpose. DNA was extracted from reference tissues of eight species considered to be the highest risk of strike by the ATSB. These are listed in table 1.

Table 1: List of ATSB high risk species from which reference DNA was extracted and sequenced for the DNA databank

Common Name	Scientific name	Tissue Reference	ATSB Hazard Ranking
White-bellied Sea-eagle	*Haliaeetus leucogaster*	EBU9977	1
Australian White Ibis	*Threskiornis mollucca*	EBU2557	2
Australian Wood Duck	*Chenonetta jubata*	MV3422	3
Little-red Flying-fox	*Pteropus scapulatus*	EBU12511	4
Spectacled Flying-fox	*Pteropus conspicillatus*	EBU26167	4
Galah	*Cacatua roseicapillus*	EBU11322	5
Silver Gull	*Larus novaehollandiae*	U37301	6
Black Kite	*Milvus migrans*	EBU12262	7

Once DNA was extracted, fragments from one to four mtDNA genes were amplified and sequenced (detailed methods in appendix 2).

2.1.3 DNA sampling kit

A DNA sampling kit was developed for distribution to aerodromes. The kit contained the following items:

- 1x pair gloves, 2x zip lock bags, 1x instruction sheet, 1x sterile DNA swab, 1x sterile pad, 1x tube of sterile water.

The purpose of the kit was to provide adequate instructions and sterile materials to aerodrome personnel who would be involved in sampling strike remains. There are several reasons for this. It is essential that samples be taken in a sterile manner to reduce the chances of contamination by other sources of DNA. Also, there was a safety aspect to the kits and their instructions as there are several diseases associated with birds and bats that can be transmitted to humans if appropriate precautions are not observed. An example of the kit and instruction sheet can be found in appendix 3.

Approximately 250 sampling kits were distributed to aerodrome personnel around Australia. The majority (~180) of these kits were distributed at the November 2005 meeting of the Australian Airports Association in Hobart, Tasmania. The remainder were sent to aerodrome personnel upon individual request.

2.1.4 DNA from strike samples

Samples were placed at -80ºC upon receipt and before DNA was extracted each sample was photographed. Commercial DNA extraction kits UltraPure (distributed by Geneworks for Mo Bio) and QIAamp micro (distributed by Qiagen) were used for this purpose, followed by amplification of mtDNA gene fragments and sequencing (see appendix 2).

2.2 Feather microscopy

Feather experiments were conducted to examine feather morphology at high magnification. At high levels of magnification, specific regions of the contour feathers display characteristics that are distinct to each species. A feather databank of high risk species was initiated using both light microscopy and scanning electron micrography.

The light microscope images were taken with a Spot Flex digital camera through a Leica MZ16 stereo microscope.

The following methods were used for derivation of the Scanning Electron Micrographs:

1) The feathers were cleaned in acetone, sonicated for 1 minute, then air dried in a filter paper lined glass dish.

2) Sections of the feather (contour and downy) were removed and mounted on an aluminium stub with a carbon double sided tab.

3) Stubs were then Gold Sputter Coated.

4) Feathers were examined and imaged using a LEO 435VP Scanning Electron Microscope with a Robinson Backscattered Electron detector.

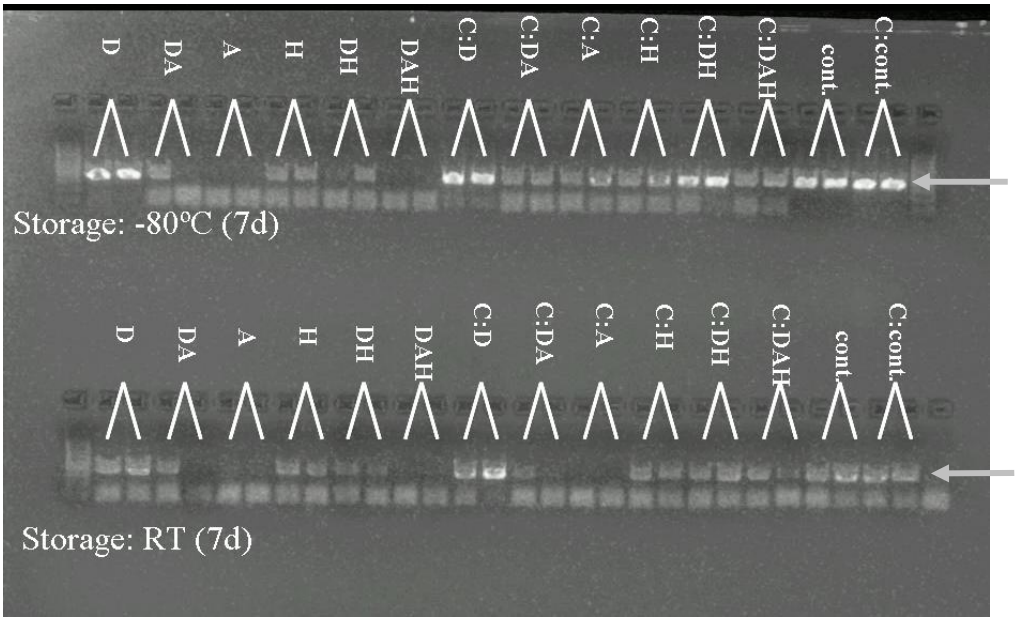
5) Micrographs were created from a mix of Secondary and Backscattered electron signals.

3 RESULTS

3.1.1 Test tissues

The test tissue experiments involved subjecting replicate samples of chicken liver to four extreme environmental conditions (heating, drying, autoclaving and carbon) and determining if these were still able to be used for DNA identification purposes after being stored at optimal (-80ºC) and sub-optimal (RT) temperatures. Test tissue amplification results (figure 1) indicate the most damaging conditions are autoclaving and leaving the sample at RT for 7 days. The other conditions (drying, heating and carbon) did not affect the samples compared to the treatments of autoclaving and 7 days at RT.

Figure 1: Amplification of Cytochrome *b* gene from test tissues subject to varying conditions (D: drying, A: autoclaving, H: heating, C: carbon)



The genes chosen for amplification in this experiment are typically easy to amplify. The top band (indicated by grey arrows in figure 1) indicates successful amplification. As a result, failure to amplify, as is the case for the ‘A’ and ‘DAH’ treated samples, strongly indicates the DNA from these test samples is damaged beyond amplification.

3.1.2 DNA from high risk species

DNA was extracted[4] for the species listed in table 2 and fragments of the mitochondrial genes 12S, 16S, Cytochrome *b* (Cyt *b*) and/or Cytochrome Oxidase I (COI) amplified[5] and sequenced for the purpose of creating a DNA databank[6].

Table 2: List of species from which reference DNA was extracted for the DNA databank and those mtDNA genes that were successfully amplified and sequenced

Common Name	Scientific name	Tissue Reference	ATSB Hazard Ranking	12S gene	16S gene	Cyt *b*	COI
White-bellied Sea-eagle	*Haliaeetus leucogaster*	EBU9977	1			✓	
Australian White Ibis	*Threskiornis mollucca*	EBU2557	2		✓	✓	✓
Australian Wood Duck	*Chenonetta jubata*	MV3422	3			✓	
Little-red Flying-fox	*Pteropus scapulatus*	EBU12511	4	✓	✓	✓	✓
Spectacled Flying-fox	*Pteropus conspicillatus*	EBU26167	4	✓	✓	✓	✓
Galah	*Cacatua roseicapillus*	EBU11322	5	✓	✓	✓	
Silver Gull	*Larus novaehollandiae*	U37301	6			✓	
Black Kite	*Milvus migrans*	EBU12262	7			✓	

These genes were chosen as they are all diagnostic to species and sequence readily. Cytochrome Oxidase I is used as the 'DNA barcoding' gene for animal species and 12S, 16S and Cytochrome b are also a useful mtDNA genes for identification of birds and mammals to species.

3.1.3 DNA sampling kits and DNA from strike samples

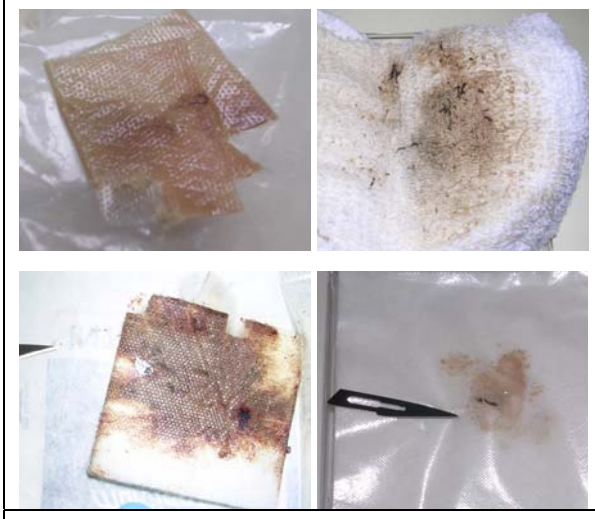
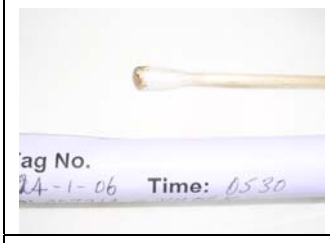
A total of 29 aircraft strike samples were received throughout the project duration (July 2005 to June 2006). The 29 samples were from aerodromes in five different Australian states (see table 3). These included blood, tissue (including muscle, intestines and skin) and feathers from strikes that were not able to be identified via traditional methods (due to the lack of a carcass, see also examples in figure 2).

4 The majority of tissues (ie muscle, heart, blood, and liver) contain high quantities of DNA. A commercial DNA extraction kit was used to extract DNA from these tissues.

5 A technique called the Polymerase Chain Reaction (PCR) is routinely used to isolate just one gene from a whole genomic DNA extraction. This method of isolation results in the creation of many copies of this gene, hence the term 'amplified'.

6 To create the DNA databank, DNA was extracted from voucher specimens and amplified regions from specific genes.

Figure 2: Examples of the variety of aircraft strike samples received throughout the project that could not be identified morphologically

	Blood sampled using the absorbent pad provided with the DNA sampling kit.
	Blood sampled using the sterile swab provided with the DNA sampling kit.
	Feather fragments received.
	Some samples received were a combination of blood and other tissues.

All samples were able to be identified to a species. Of the 29 samples, six were bats, 22 were birds and one was a human contaminant (table 3). Only seven of the samples were identified as high risk species. These included four bat samples (from two Flying-Fox species) and two birds (two species, Galah and Pacific Black Duck). The 15 remaining bird and one bat species were considered lower risk by the ATSB. Common names marked by an asterisk (*) indicate samples from species identified by the ATSB as the eight highest risk.

Table 3: Detailed list of strike samples

Sample#	Location	Species (common name)	Suspected species	Correct id.?	Tissue type	DNA kit used?
1	Qld	*Pteropus conspicillatus* (Spectacled Flying fox)*	Unknown	-	Blood, skin	N
2	Qld	*Pteropus scapulatus* (Little Red Flying fox)*	Unknown bat	-	Intestine	N
3	Qld	*Merops ornatus* (Rainbow Bee-eater)	Medium bird	-	Blood	Y
4	Qld	*Gymnorhina tibicen* (Australian Magpie)	Unknown bird	-	Blood	Y
5	NT	*Stiltia Isabella* (Australian Pratincole)	Swift	No	Blood, feathers	Y
6	WA	*Falco cenchroides* (Nankeen Kestrel)	Kite	No	Feathers	Y
7	NSW	*Cacatua roseicapilla* (Galah)*	Galah	Yes	Blood, feathers	Y
8	Qld	Contaminant	Unknown medium bird	N/A	Sample contaminated with saliva from maintenance engineer	N
9	Qld	*Apus pacificus* (Fork-tailed Swift)	Unknown bird	-	Blood, feather	Y
10	Qld	Turnix spp. (Button Quail)	Unknown small bird	-	Blood, skin, muscle, feather	Y
11	Qld	Charadrius lechenaulti (Greater Sand Plover)	Unknown bird	-	Feather	Y
12	*Qld*	*Petrochelidon ariel (Fairy Martin)*	*Unknown medium bird*	*-*	*Blood*	*N*
13	*Qld*	*Apus pacificus (Fork-tailed Swift)*	*Welcome swallow*	*No*	*Blood, feather*	*Y*

Table 3: Continued

Sample#	Location	Species (common name)	Suspected species	Correct id.?	Tissue type	DNA kit used?
14	Qld	*Pipistrellus adamsi* (Cape York Pipistrelle)	Unknown bird	No	Blood	Y
15	Qld	*Apus pacificus* (Fork-tailed Swift)	Unknown medium bird	-	Blood	Y
16	NSW	*Hirundapus caudacutus* (Spine-tailed Swift)	Spine tailed swift	Yes	Entire body	Y
17	Qld	*Mirafra javanica* (Australian Lark)	Unknown bird	-	Blood	Y
18	Qld	*Miniopterus australis* (Little Bentwing Bat)	Unknown bird	No	Blood	Y
19	Vic.	*Corvus cornoides* (Australian Raven)	Magpie	No	Blood	Y
20	WA	*Gymnorhina tibicen* (Australian Magpie)	Sparrow	No	Blood	Y
21	Qld	*Pteropus scapulatus* (Little Red Flying-fox)*	Flying fox	-	Blood, skin, bone	Y
22	Qld	*Pteropus scapulatus* (Little Red Flying-fox)*	Unknown bird	No	Blood	Y
23	Qld	*Charadrius ruficapillus* (Red-capped Plover)	Unknown bird	-	Feather	N
24	Qld	*Burhinus grallarius* (Bush Stone Curlew)	Unknown bird	-	Feather	N
25	Qld	*Petrochelidon ariel* (Fairy Martin)	Unknown bird	-	Feather	N
26	Vic.	*Elanus auxillaris* (Black-shouldered Kite)	Magpie	No	Blood, muscle	Y
27	NT	*Falco cenchroides* (Nankeen Kestrel)	Kite	No	Blood, feathers	Y
28	Qld	*Falco cenchroides* (Nankeen Kestrel)	Kestrel	Yes	Blood, feathers	Y
29	Vic.	*Anas superciliosa* (Pacific Black Duck)*	Pacific black duck	Yes	Blood, feathers	Y

3.1.4 Feather microscopy

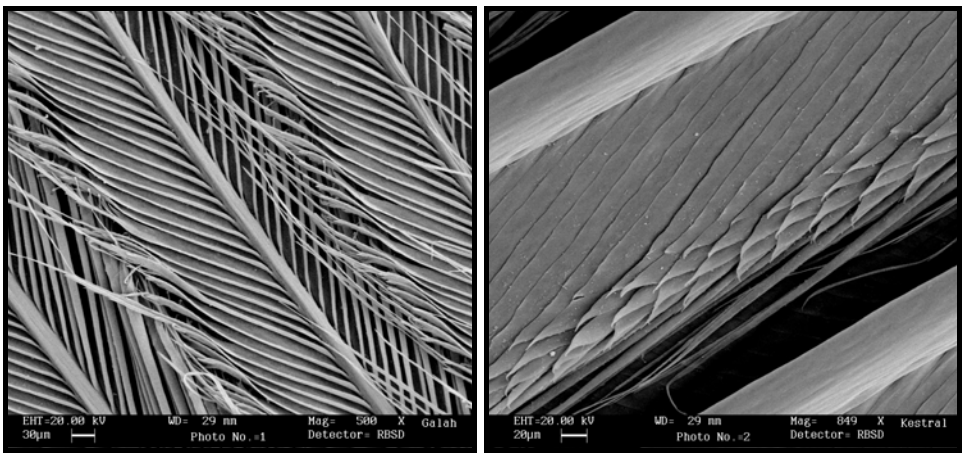
Feather experiments were conducted to examine feather morphology at high magnification. A feather databank of high risk species was initiated, see table 4 for a list of species. Analysis of contour feathers for these species show distinct differences between species (see figure 3 and figure 4 for examples).

Table 4: List of species used for the feather microscopy databank

Common Name	Scientific name	Feather Reference	ATSB Hazard Ranking
White-bellied Sea-Eagle	*Haliaeetus leucogaster*	O.45392	1
Australian White Ibis	*Threskiornis mollucca*	O.57510	2
Australian wood duck	*Chenonetta jubata*	O.47641	3
Galah	*Cacatua roseicapillus*	EBU11322	5
Silver gull	*Larus novaehollandiae*	O.47929	6
Black kite	*Milvus migrans*	EBU12262	7
Collared Sparrow Hawk	*Accipiter cirrhocephalus*	N/A	8
Nankeen Kestrel	*Falco cenchroides*	N/A	N/A

Figure 3 shows obvious differences in the contour[7] feathers of a Galah and a Nankeen Kestel at 20-30μm. Figure 4 shows a higher magnification (10μm) view of the plumulaceous[8] barbs, which are found at the downy base of contour feathers for both galah and kestrel.

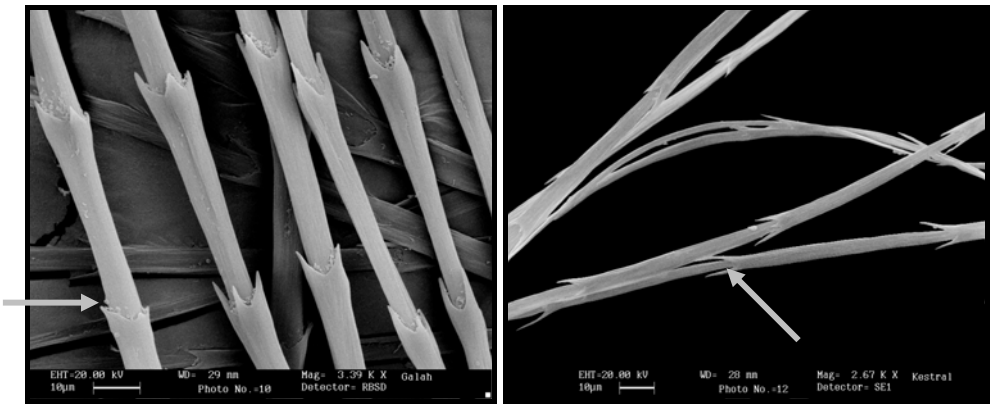
Figure 3: Scanning electron micrographs of contour feathers from Galah (left) and Nankeen Kestrel (right) - scale 20-30μm



7 Contour feathers are the outermost feathers of a bird and form the visible body, wing and tail plumage.

8 Plumulaceous (or downy) barbs are found at the base of contour feathers. When viewed at high magnification these plumulaceous barbs display specific micro-characters that are often distinct to each species.

Figure 4: Scanning electron micrographs of plumulaceous barbs from Galah (left) and Nankeen Kestrel (right) contour feathers - scale 10μm - Expanded nodes are highlighted by the arrows



Plumulaceous barbs are particularly informative structures for species identification (Dove, 1997; Laybourne & Dove, 1994). Characters such as the shape of the expanded nodes (highlighted by the arrows above) and the length between the nodes are often distinct to a bird species.

4 DISCUSSION

The DNA-based experiments carried out in this study included testing tissue DNA damage and actual strike samples. In addition, DNA was extracted from vouchered museum specimens for the purpose of creating a reference DNA databank of high risk species.

Results from the experiments involving test tissues showed that despite the application of conditions that are assumed to be damaging to DNA, DNA was still able to be extracted and successfully analysed from the majority of samples. The treatment that caused the most damage was autoclaving, which involves high temperature and high pressure for an extended period. Autoclaving is known to destroy the majority of DNA, so this result is not surprising. In addition, it is unlikely that the conditions on an aircraft would be as extreme as autoclaving. It is more likely that the conditions would be more similar to the other treatments (drying, heating and carbon), which do not appear to do irredeemable damage to DNA. The other most damaging condition that was found to affect the quality of DNA was if the sample was left at RT for an extended period of time (7+ days). This is an important result for collection under the conditions present on an aircraft (where the sample is collected off a non-sterile surface) as it highlights the necessity to freeze these samples as soon as possible after collection to preserve as much DNA as possible for successful analysis.

The DNA databank was established using the ATSB top eight high risk species. Interestingly, from the 29 unidentified strike samples received only 27 per cent of samples came from this list. The remaining >70 per cent of samples were from birds or bats either not listed, or ranked much further down the list. Although the 29 samples represented a relatively modest sample size and are biased to samples not readily identified by other means, the trend shown indicates it would be prudent to consider a very wide range of species in assessing which birds and bats are likely to be involved in collisions with aircraft.

The DNA sampling kits (see appendix 3) were widely distributed to major and regional aerodromes and were used on 22 occasions for sampling. Samples returned with the kits included blood, tissue (including muscle, intestines and skin) and feathers. Once used for sample collection, the kits were returned and DNA was then extracted from the samples 100 per cent of the time.

There were seven samples returned that did not use the kits. Of these, there were three were partial feather samples that had previously been analysed by external experts but were unable to be identified morphologically. We were able to successfully extract DNA from these difficult samples and identify them to species. The other notable sample collected without the use of a DNA sampling kit returned a human contaminant. Further investigation revealed that an aircraft engineer had moistened the sample with saliva as it was very dry, which contaminated the sample with human DNA. Had a DNA sampling kit been used on this occasion it is likely this would have been avoided providing the enclosed instructions were followed.

Another interesting trend suggested by the strike samples indicate that identifications based on animal parts by aerodrome staff may not always be accurate. Collectors were asked to provide a putative identification, if possible, on the collection slip (see appendix 3; figure 6). On the 14 occasions that putative identifications were provided, DNA analysis revealed 10 (or 71 per cent) to be incorrect. Based on our strike samples (which were predominantly ‘unknowns’) little weight can be given to this error rate. Certainly, it is more difficult to identify only part of an animal and collectors may have been more likely to record a strike as unknown to the ATSB rather than include a putative species (as requested on our collection sheet). Nevertheless, our results indicate that a diverse range of species are involved in aviation strikes and that incorrect identifications or biased estimates of strike frequency could seriously impact decisions covering the management of problem species.

Analysis of the feather microscopy images revealed that high magnification of different sections of contour feathers showed morphological characteristics that appeared to be diagnostic to species. Even at 20-30μm scale feathers were distinct from one another (for example see figure 3). In addition, at higher magnifications (10μm) the expanded nodes present in the plumulaceous (downy) barbs showed distinct differences in the shape and regularity of the expanded nodes (for example see figure 4). The only limitation with this technique is that expert knowledge in comparison of these morphological characteristics is required for definitive identification to species.

5 CONCLUSIONS

The primary aim of this project was to investigate the feasibility of using forensic DNA testing to identify wildlife strikes. Using techniques developed in the laboratory, the identification of strike samples, that otherwise would have been recorded as unknown due to lack of morphological characters, were developed.

Based on experience gained through this project, the following actions are suggested:

1) Tissue experiments showed the most damaging conditions to DNA occur if the sample is left at room temperature for 7+ days. If DNA testing were to be routinely used to identify unknown strike samples, appropriate procedures should be followed during sample collection to avoid extended non-refrigerated storage to ensure the greatest chance of a positive DNA result.

2) It was expected that the ATSB's top eight highest risk species to be the animals most frequently involved in strikes. Interestingly, only 27 per cent of the unidentified samples received were species from this list. Overall, 20 different species, including three bat species and 17 bird species, were detected using DNA identification of strike samples. Given the greater than expected diversity of species involved in strikes, the DNA databank should be expanded to include a broader range of relevant bird and mammal species.

3) The diversity of bird and mammal species involved in aviation strikes is likely to vary with habitat, climate and season. Ideally, the impact of high risk species should be assessed based on a more localised geographic scale and include assessment of seasonal variation.

4) Strike samples returned to the laboratory using the DNA sampling kit indicate that identifications based on animal parts by aerodrome staff may not always be accurate. If this error rate is even slightly reflected in the daily collection of bird strike data this runs the risk of recording an incorrect species responsible for a strike. It would seem prudent to provide aerodromes with further assistance with species identification to ensure that the correct species are being managed in habitats surrounding civilian aerodromes.

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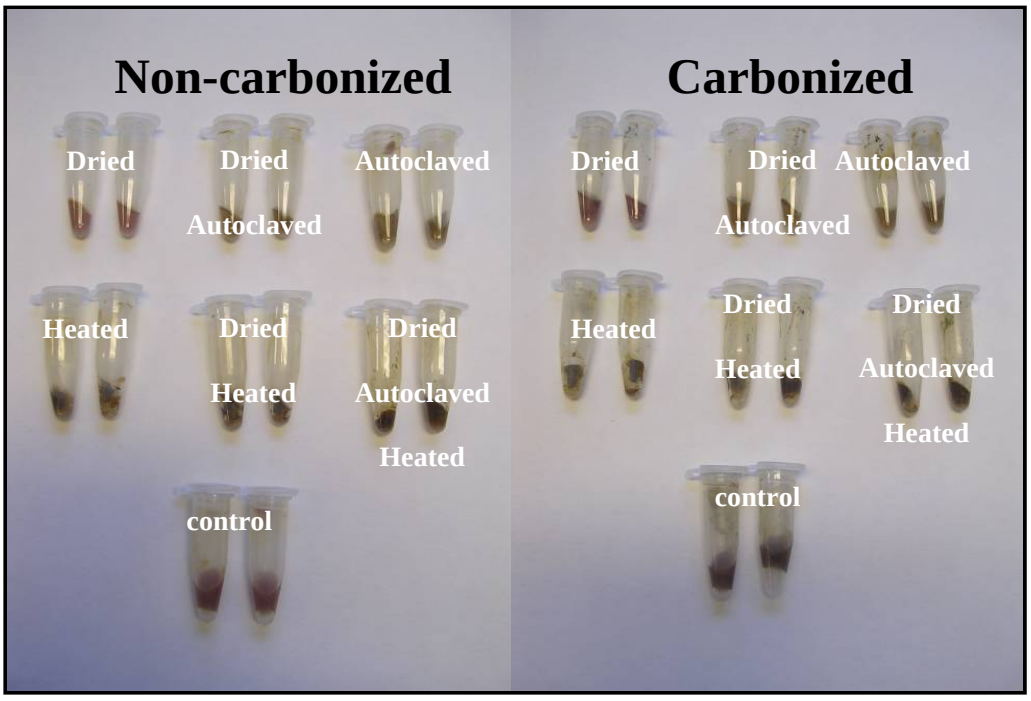
7 APPENDIXES

7.1 APPENDIX 1: Test tissue conditions

Table 5: Description of treatment conditions for each sample and replicate - '+' indicates treatment for that column was carried out '-' indicates treatment for that column was not carried out (below is a pictorial representation of the same)

Sample#	Drying	Autoclaving	Heating	Carbon
1,2	+	-	-	-
3,4	+	+	-	-
5,6	-	+	-	-
7,8	-	-	+	-
9,10	+	-	+	-
11,12	+	+	+	-
13,14	+	-	-	+
15,16	+	+	-	+
17,18	-	+	-	+
19,20	-	-	+	+
21,22	+	-	+	+
23,24	+	+	+	+
25,27	-	-	-	-
26,28	-	-	-	+



7.2 APPENDIX 2: PCR and sequencing conditions

PCR amplifications were carried out in 25µl volumes containing 1.5mM $MgCl_2$, 0.025mM of each dNTP, 12.5pmol of each primer, 0.2units of Qiagen Taq DNA polymerase, 2.5µl of Qiagen 10x PCR buffer and 1-100ng of whole genomic DNA. A negative control (containing no DNA template) is included for each batch of amplifications. The following cycling profile is used initially for all experiments: initial denaturation at 94˚C for 1 minute, then 30 cycles of 94˚C for 20 seconds, annealing for 45 seconds and extension at 72˚C for 1.5 minutes, and a final extension at 72˚C for 5 minutes. PCRs are checked by running 5µl (or 20 per cent) of the reaction on a 1.5 per cent agarose gel. For primer sequences see table 6).

Successful PCRs are purified using the AMPURE PCR purification system available from Agencourt Bioscience Corporation (distributed by GeneWorks Pty Ltd in Australia). Purified PCRs are then quantified by running 1-5µl (or 3 to 6 per cent) on a 1.5 per cent agarose gel. Both forward and reverse strands are sequenced using ½ volume DYEnamic ET terminator premix (distributed by Amersham Biosciences for GE Healthcare) with the same primers used for PCR according to manufacturers' instructions. The samples are purified using the ethanol and ammonium acetate purification then run on a MegaBACE 500 Genetic Analyser. Forward and reverse strands are combined and sequences checked for errors using the computer program Sequencher (Genecodes). Only samples that provide clear and unambiguous sequence are subject to further analyses.

Table 6: Primer sequences, annealing temperatures and references (the same primers are used for both amplification and sequencing)

Gene	Primer name	Annealing temp.	Sequence 5' → 3'	References
12S	12SrDNA-F	53˚C	TGA CTG CAG AGG GTG ACG GGC GGT GTG T	Kocher et al. (1989)
12S	12SrDNA-R	53˚C	AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC	Kocher et al. (1989)
12S	12S-f	53˚C	GCA GCT TCC GCT ACK YCT AC	Julie Macaranas (2000)
12S	12S-r	53˚C	GTG CCA GCA GTC GCG GTC A	Julie Macaranas (2000)
16S	16S-f	53˚C	CGC CTG TTT AAC AAA AAC AT	Miya and Nishida (1996)
16S	16S-r	53˚C	CCG GTC TGA ACT CAG ATC ACG T	Miya and Nishida (1996)
Cytochrome oxidase I	COI-Mam-1f	53˚C	CATGCATTCGTAATAATC TTC	Designed by R.Johnson AM DNA laboratory
Cytochrome oxidase I	COI-Mam-2r	53˚C	ATCATTTGATATTGCCTC CGTG	Designed by R.Johnson AM DNA laboratory
Cytochrome *b*	M5	50˚C	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	Kochner and White (1989)
Cytochrome *b*	M6	50˚C	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A	Kochner and White (1989)

7.3 APPENDIX 3: DNA sampling kit

Figure 5: Photograph of a DNA Sampling Kit developed for this project

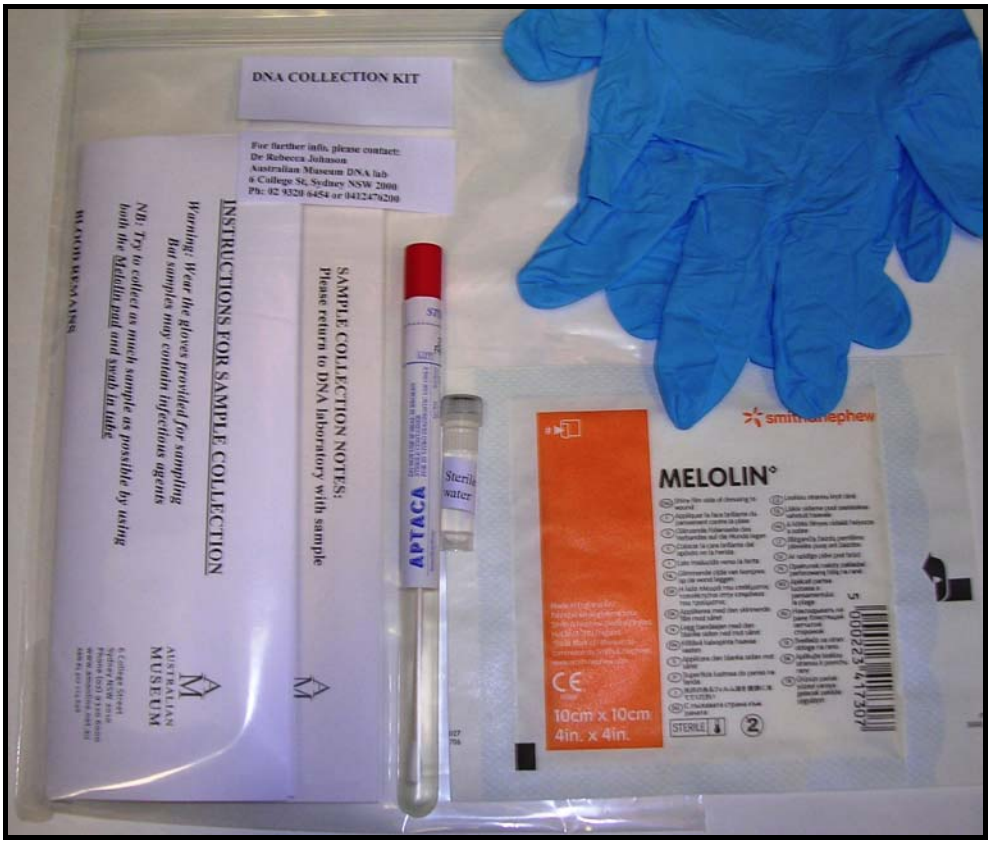


Figure 6: Instructions for sample collection from DNA sampling kit

INSTRUCTIONS FOR SAMPLE COLLECTION

Warning: Wear the gloves provided for sampling
Bat samples may contain infectious agents

NB: Try to collect as much sample as possible by using both the Melolin pad and swab in tube

BLOOD REMAINS

1) If dry, dip sterile swab in sterile water (provided) and swab over sample
2) Place swab back in sterile tube
3) Send entire swab in tube in plastic bag
4) Send by air freight using enclosed label

NB: BE VERY CAREFUL NOT TO TOUCH SWAB TIP WITH ANYTHING OTHER THAN THE SAMPLE TO BE COLLECTED – TO ENSURE STERILE CONDITIONS

MOIST/FLESHY TISSUE REMAINS

1) If moist tissue remains, wipe up as much as possible with the Melolin pad
2) Double bag Melolin pad
3) Send by air freight using enclosed label

FEATHERS ONLY

1) If whole or partial bird, **pluck** (DO NOT CUT) a variety of feathers (e.g. breast, back, wing, tail) especially those with obvious colour pattern.
2) Place feathers or feather remains in plastic collecting bag,
3) Send by air freight using enclosed label

Cut this portion off and stick on envelope for returning to DNA laboratory:

REBECCA JOHNSON
AUSTRALIAN MUSEUM
DNA LABORATORY
6 COLLEGE STREET
SYDNEY, NSW, 2000

SAMPLE COLLECTION NOTES:
Please return to DNA laboratory with sample

Name of collector:

Date and time of collection:

Other information (e.g. condition of sample, suspected species, time of strike, time of sampling):