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Stephen D. Sarre
Institute for Applied Ecology and the Invasive Animals Cooperative Research Centre, University of Canberra, Australia

Rachel Walsch
Institute for Applied Ecology and the Invasive Animals Cooperative Research Centre, University of Canberra, Australia

Niccy Aitken
Institute for Applied Ecology and the Invasive Animals Cooperative Research Centre, University of Canberra, Australia

Alison Foster
Tasmanian Department of Primary Industry and Water, Tasmania

Nick Mooney
Tasmanian Department of Primary Industry and Water, Tasmania

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STEPHEN D. SARRE, RACHEL WALSH AND NICCY AITKEN, Institute for Applied Ecology and the Invasive Animals Cooperative Research Centre, University of Canberra, Australia
ALISON FOSTER AND NICK MOONEY, Tasmanian Department of Primary Industry and Water, Tasmania

Abstract: The red fox (*Vulpes vulpes*) has wreaked havoc on mainland Australia's environment and agricultural production since its introduction in the 1870s. Over the same period, the southern Australian island State of Tasmania has remained virtually fox-free, allowing its unique biodiversity to remain relatively pristine. Recently, an unknown number of foxes were deliberately or accidentally introduced to Tasmania. Some of those animals and possibly their progeny now live in the wild in Tasmania. Finding foxes in a state the size of Tasmania presents special problems for wildlife managers, but is essential to prevent their establishment in this stronghold for Australian marsupials. To assist in finding foxes in Tasmania, we have developed DNA detection approaches specifically for foxes that utilize the ubiquitous mitochondrial DNA found on the surface of mammal scats. Using these approaches, fox DNA has been detected in three different regions in Tasmania and have provided the basis for intense control efforts in those areas. We are now expanding our approach to include other predatory mammals of interest (including both native marsupials and other introduced mammals) and increasing the breadth and scope of our surveys.

Key Words: eradication, DNA-based detection, invasive species, mitochondrial DNA, red fox, scat surveys, Tasmania, *Vulpes vulpes*.

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INTRODUCTION

Biological invasions have been a major cause of worldwide species endangerment and extinction during the 19th and 20th centuries (Caughley 1994). Island fauna in particular have suffered heavily when exposed to novel competitors or predators (Corke 1987, Burbidge and Manly 2002). Australia, owing to its long isolation is sometimes viewed as an island continent, and has a highly distinctive biota, but it has the worst record for mammalian extinctions globally, with 27 recorded since 1780 (Short and Smith 1994). A major cause of species loss has been the predatory red fox (*Vulpes vulpes*) which was introduced to Australia from Europe in 1855 for hunting, and is now a devastating invasive pest to both wildlife and agriculture (Short and Smith 1994). Foxes have been implicated in the decline of several mainland and island species of Australian mammal, and are listed as a threatening process under the Australian Environment Protection and Biodiversity Conservation Act 1999.

In contrast to most of Australia, the native fauna of Tasmania, a large island state of 68,332 square km, some 200 km to the south of the Australian mainland, is relatively unchanged by European settlement and has seen the known extinction of only one mammal in historical times (the thylacine). Tasmania's isolation has meant that it has been spared predation by the fox and, as a consequence, now represents a living repository of pre-European Australian marsupial communities that is unparalleled elsewhere.

In September 2001, a fox was shot in northern Tasmania and there were reports of a second shot in the midlands, near Conara. In 2003, the fresh remains of a fox were found near Burnie on Tasmania's northern coast and two more carcasses (one at Lillico Beach near Devonport in December 2005 and a second at Cleveland/Conara in the midlands in August 2006) have been found (Figure 1). These foxes are believed to have been part of, or the progeny of, a deliberate introduction of an unknown number of foxes to the island around 1999/2000 (Saunders et al. 2006). The risk that this introduction poses to Tasmania's wildlife (Saunders et al 2006), and its tourism and pastoral industries led to the establishment of a taskforce by the Tasmanian and Australian federal governments to define and manage the threat.

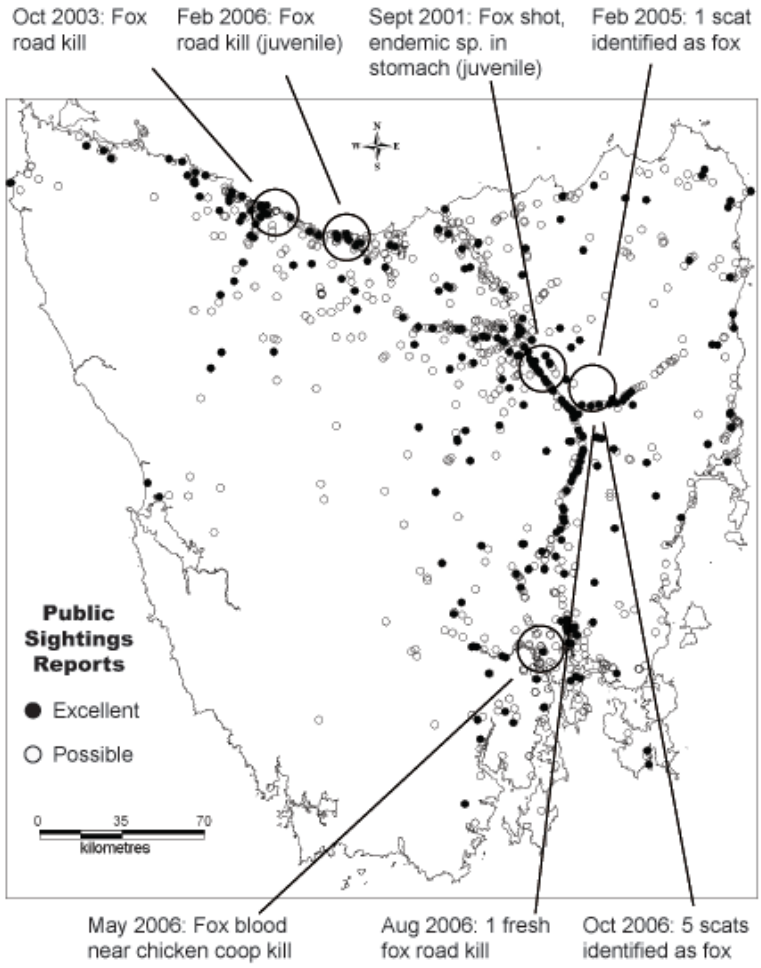

Figure 1. Fox sightings reports in Tasmania since 2001 and the sites of scat identified as positive by DNA testing. Fox sightings are focussed around centres of human habitation and along major highways which is likely to present a bias in sampling rather than a true indication of fox activity. Proposed sampling for scats in the future will be directed at a systematic collection across the state.

TACTICAL MONITORING APPROACHES

Over 1,500 fox sightings have been reported by the public since 2002 (Figure 1), suggesting that the fox may already be widespread on the island. However, most reported sightings have been difficult to verify, and many are believed to be erroneous. This lack of unequivocal evidence has led to public scepticism about the threat posed by foxes and consequent political pressure to reduce funding on attempts to monitor and control the invasion.

The control program implemented by the Tasmanian Fox Eradication Program has been directed towards tactical baiting with the poison 1080 (sodium monofluoroacetate) which can be toxic to native fauna. In order to minimise non-target deaths (particularly given the rich fauna of medium sized marsupial carnivores), baits must be set by hand, which is expensive and time consuming. Currently, baiting is centred on regions where reported sightings of foxes have been most concentrated or where hard evidence (e.g., road-kill carcasses) has been found. Accurate detection and monitoring of the fox is critical to the efficacy of such control operations. However, given the likely high incidence of error, the dispersed nature of the sightings and the fact that they are concentrated around regions of high human activity such as major highways, this targeting can be at best approximate and its effectiveness difficult to determine.

The biased and uncertain nature of public sightings requires that other means of monitoring this elusive predator be developed. One approach that does not rely on ad hoc, fleeting and usually

nocturnal sightings, is the collection of predator scats (faeces). Canid scats can persist in the field for weeks or even months (Kohn et al. 1999), and several species, including foxes, tend to leave them in prominent places such as along trails (MacDonald 1980). To bolster evidence from reported sightings, earlier phases of this program employed analysis of faecal (scat) morphology and hair to define fox presence. The main advantages of such surveys is that they can be done by relatively untrained personnel and provide exact location data for targeted control. The disadvantage is that definitive morphological diagnosis can be difficult in a place like Tasmania where there are at least 6 major mammalian predators that can produce scats of similar size and shape. Diagnostic hairs associated with scats, can provide additional information about the species of origin, but occur in only about 10% of scats.

DEVELOPMENT OF A DNA TEST FOR FOXES

DNA analysis of scats provides a solution to this problem, by combining the benefits of sampling offered by scats with the robust detection provided by approaches based on the polymerase-chain-reaction (PCR, Kohn and Wayne 1997). These approaches have been primarily used to study the ecology of native wildlife. However, the same principles can be applied to the detection and mapping of elusive invasive species, such as the introduced red fox. With appropriate verification of the methodology ((Taberlet and Luikart 1999), non-invasive DNA-based methods could provide the high-quality distribution data that is required for effective control.

To this end, we have developed a PCR-based test specific to foxes that excludes the amplification of other carnivore DNA and provides a rapid initial screen of all scats collected. Further verification is required following the initial identification of a scat positive for fox DNA. The full details of this test and its development can be found in Berry et al. (2007). The key points of the test and its development are as follows.

1. The test involves a single multiplex PCR that amplifies fragments from two genes of the mitochondrial DNA genome. The first fragment targeted is a portion of the 12 rRNA gene, and is designed to amplify in the presence of DNA from any carnivore present in Tasmania. The amplification of this fragment acts as a control for the successful extraction of DNA from the scat. The second fragment amplified is from the cytochrome b gene and targets fox DNA specifically.
2. Field testing demonstrated that our approach to detecting fox DNA in scats was 100% successful in scats up to 12 weeks old despite significant loss of template DNA through degradation. It is quite likely that a high rate of detection is possible for scats that are considerably older than 12 months.
3. The test was implemented by the Department of Primary Industry and Water in 2004 for scat identification activities on contract to the Wildlife Genetics Laboratory at the University of Canberra. Following the initial implementation, additional analytical procedures were added to the test to minimise the risk of false positive identifications of fox traces (see below).
4. The positive identification of scat or other trace material now acts as a trigger for the focus of fox eradication actions.

IMPLEMENTATION OF THE DNA TEST FOR TASMANIAN FOX ERADICATION

The implementation of the test for samples derived from Tasmania necessitated the development of a series of protocols to maximise the value of each sample collected and to minimise the risk of Type 1 errors (false identification of a sample as fox when it is not) or Type 2 errors (failure to detect a fox scat). The minimisation of these risks falls into three general categories: (1) sample handling methods that minimise the loss of DNA from a sample once it had been collected and, therefore, minimise the risk of not identifying a scat as from fox when it is one (Type 2 error), (2) strategies that minimise the risk of sample contamination from collection to analysis (Types 1 and 2 errors), and (3) strategies that minimise the risk of false positives for foxes (Type 1 errors). Below, we record the approaches taken to minimise these risks. A fourth category of risk minimisation relates to the probability of a fox scat not being found should it actually be in an area surveyed and the probability that an area containing fox scats is not searched at all. Given the immense area of suitable habitat for foxes in Tasmania (>30,000 km^2) the risk of not searching an area containing foxes is substantial and its minimisation requires a strategic and systematic approach to survey. That

approach is currently under development and will not be detailed here.

Sample Handling to Minimise the Loss of DNA from a Sample

DNA in the epithelial and other cells attached to scats will start to deteriorate immediately as cellular enzymes (nucleases) start to degrade the DNA. Large DNA fragments will be cut into smaller and smaller pieces by this process. Two key characteristics of the scat will influence the rate at which DNA degradation occurs are the amount of moisture retained in the cells and the temperature to which the scat is exposed (Murphy et al. 2002). Low moisture content will retard enzyme activity and reduce DNA degradation that may occur through hydrolytic action and cool temperatures will slow the rate of degradation by reducing biological activity. The rate of degradation of the DNA on and within scats can therefore be minimized by removing moisture from the scat and keeping them cool. Therefore, our approach has been to dry the scats as quickly as possible following collection. An alternative approach would be to freeze the scats or preserve them in ethanol, but this makes air transport difficult and expensive (airline companies often do not like transporting dry ice or flammable material) and creates storage problems if the scats are to be stored for a long time. As a consequence we have adopted the following approach:

1. All scats are collected in non-greased paper bags and sealed at the point of collection. This allows air to circulate through and around the scat and begin the drying process.
2. All scats are then dried in a drying room in Launceston, Tasmania, where temperatures are maintained at around 25^{o}C (±5^{o}C).
3. An electric fan and dehumidifier are used to maintain airflow and decrease air moisture in the drying room. Drying takes 2-3 days.
4. Once dry, scats are packed in cardboard boxes and mailed for DNA analysis.
5. Following analysis, scat samples are archived at the CSIRO Australian National Wildlife Collection for future work which may include further species identification or DNA-based dietary analyses.

Minimising the Risk of Sample Contamination at Collection

The introduction of foreign DNA (including human) may compromise the ability for the DNA of the target species to be amplified in the laboratory and, hence, reduce the probability that fox DNA is detected in a scat should it be present. As a consequence, it is important that scats are not handled directly by the collectors. There is also a significant risk of disease transfer to scat collectors so occupational health and safety concerns dictate a no handling policy as well. Our approach is that scats must be picked up using either disposable latex gloves or ‘chopsticks’ improvised from vegetation. New sticks or gloves are used for each scat to minimise the risk of cross-contamination. Scats and animal parts are not allowed to touch personnel, clothing or any equipment other than the sticks or disposable gloves and the interior of the paper bags. Used latex gloves are collected in a bag and disposed of appropriately upon return. Calico bags are used for carrying collected scats and no other material. Other signs (such as fur or blood) collected as part of the activities are stored separately to the scats. Once the scats are bagged, they are never removed from the bag and go directly to the drying room.

Minimising the Risk of False Positives for Foxes

The analysis of trace samples of DNA such as those found on animal scats includes a risk of false positives through artefacts of the PCR process. Such artefacts will generally arise from the amplification of closely related DNA sequence from DNA contaminates in the laboratory itself or from prey DNA present in the scat. The potential for laboratory-based contamination is managed rigorously through a series of measures that include the spatial separation of DNA extraction, PCR set-up, post-PCR analysis, the one-way movement of samples and laboratory technicians from DNA extraction stations to the analytical laboratory, operation in DNA-free biohazard safety cabinets, and the inclusion of negative controls at all stages of preparation to test for possible contamination. These approaches follow closely the principles that are used for DNA analysis in human forensic case work. To minimise the risk of contamination by prey items, the primers used to amplify the fox

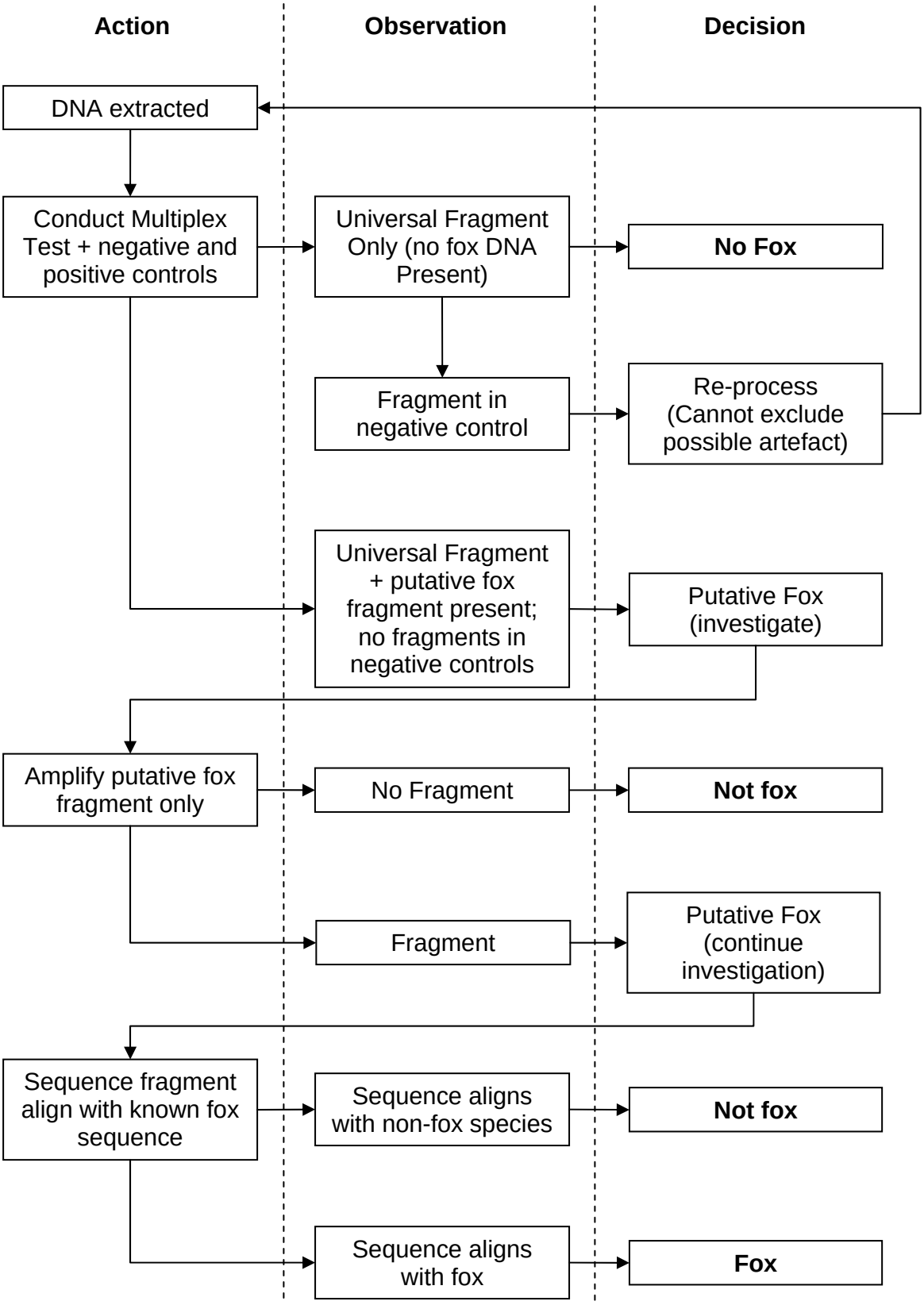

Figure 2. Flow diagram showing the sequence of tests applied before a sample can be considered to contain fox DNA. Only a sequence matching those of fox in the absence of fragments in negative controls act as a trigger for control action.

specific cytochrome b fragment in our study were designed to specifically exclude the amplification of homologous sequence from other large carnivores in Tasmania (Berry et al. 2007). However, occasionally, non-target fragments of a size similar to the target fox band are amplified and require investigation. Such fragments are sequenced and compared to sequence contained in the world-wide database GenBank (www.ncbi.nlm.nih.gov/blast/Blast.cgi) and from our own sequence records to determine the species of most likely origin. Only when a sequence from a scat matches specifically to fox do we consider a scat to be positive for fox (Figure 2).

APPLICATION OF THE FOX TEST IN TASMANIA

We have applied the test to over 2,000 scats and other trace samples collected since 2004 as part of the ongoing program to eradicate invasive foxes from Tasmania. Most scats have been collected as part of a tactical program targeting areas of fox activity as identified through reported sightings from the public. In all, we have identified six scats (from one general region, Figure 1) as containing fox DNA and have also identified a blood sample left at a chicken coop as being from fox. These findings have assisted Tasmanian authorities to target their baiting campaigns by providing solid evidence of fox presence.

A clear limitation of this 'tactical' approach used to date is that it targets areas in a biased fashion, being skewed towards areas in which most people live (and hence are likely to see foxes). In the future, the Tasmanian Department of Primary Industry and Water, the University of Canberra, and the Invasive Animals Cooperative Research Centre will conduct a systematic survey of the state for scats that will maximise the opportunity for finding fox traces and, hence, define the extent of the fox problem in Tasmania. Of central interest is how widespread foxes are. This will be used to guide the planning of strategic eradication activities. If they are widespread, then an assessment of eradication feasibility will be required.

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