Blood, sweat and tears: a review of non-invasive DNA sampling

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SUMMARY

The use of DNA data is ubiquitous across animal sciences. DNA may be obtained from an organism for a myriad of reasons including identification and distinction between cryptic species, sex identification, comparisons of different morphocryptic genotypes or assessments of relatedness between organisms prior to a behavioural study. DNA should be obtained while minimizing the impact on the fitness, behaviour or welfare of the subject being tested, as this can bias experimental results and cause long-lasting effects on wild animals. Furthermore, minimizing impact on experimental animals is a key Refinement principle within the "3Rs" framework which aims to ensure that animal welfare during experimentation is optimised. The term 'non-invasive DNA sampling' has been defined to indicate collection methods that do not require capture or cause disturbance to the animal, including any effects on behaviour or fitness. In practice this is not always the case, as the term 'non-invasive' is commonly used in the literature to describe studies where animals are restrained or subjected to aversive procedures. We reviewed the non-invasive DNA sampling literature for the past six years (346 papers published in 2013-2018) and uncovered the existence of a significant gap between the current use of this terminology (i.e. 'non-invasive DNA sampling') and its original definition. We show that 59% of the reviewed papers did not comply with the original definition. We discuss the main experimental and ethical issues surrounding the potential confusion or misuse of the phrase 'non-invasive DNA sampling' in the current literature and provide potential solutions. In addition, we introduce the terms 'non-disruptive' and 'minimally disruptive' DNA sampling, to indicate methods that eliminate or minimise impacts not on the physical integrity/structure of the animal, but on its behaviour, fitness and welfare, which in the literature reviewed corresponds to the situation for which an accurate term is clearly missing. Furthermore, we outline when these methods are appropriate to use.

KEYWORDS: eDNA, animal behaviour, fitness, refinement, animal welfare

1. INTRODUCTION

DNA data are becoming increasingly important in animal biology [1], both for experimental and observational studies. This is partially driven by the progressively cheaper and more user-friendly ways of accessing genomic information [2]. Analysis of genetic material provides data for myriad uses. In addition to analysis of phylogenetic relationships or population genetics, DNA analysis is required to determine basic information about individuals of many species [3]. When DNA analysis is required for purposes such as sexing, kinship and differentiation between cryptic species prior to experimentation with the same individuals, the DNA sampling procedure could bias the results of the subsequent experiment. It is therefore essential to minimise the effect that DNA sampling can have on the fitness or behaviour of the subject being tested. Furthermore, ethical use of animals in experimentation is guided by the '3Rs' framework of Refinement, Replacement and Reduction (e.g. [4]). The impact of DNA collection is particularly relevant to the principle of Refinement where techniques with the lowest impact on the animal model should be used whenever possible. Refinement of experimentation is only possible when impact on the animal is accurately identified.

Methods of DNA collection were originally defined as 'non-invasive' if "*the source of the DNA is left behind by the animal and can be collected without having to catch or disturb the animal*" [5,6], for example when genetic material was left behind in traces or scats (i.e. *sensu* environmental DNA (eDNA)), implicitly avoiding any impact on animal welfare.

These non-invasive DNA sampling procedures have been applied to study a wide range of animal taxa and answer various questions such as species identification, sexing, population genetics, description of the diet etc. To draw a comprehensive picture of the current use of these methods, we conducted a systematic review of the recent literature (2013-2018) and discuss what non-invasive DNA sampling is used for as well as issues relating to the misuse of the term.

2. METHOD

We conducted a keyword-based search on the Web Of Science core collection using the keywords DNA and non-invasive or DNA and noninvasive, as both spellings were originally proposed and are in common use [5,6]. We restricted our search to articles published in relevant disciplines and between 2013 and 2018. The search command used was the following:

(TS=((dna AND non-invasive) OR (dna AND noninvasive)) AND SU=(ecology OR zoology OR ornithology OR environmental sciences OR entomology OR fisheries OR behavioural science OR Biodiversity & Conservation) AND PY=(2013 OR 2015 OR 2017 OR 2014 OR 2016 OR 2018))

Results were then refined to experimental papers written in English. On the 9th of July 2018, this search yielded 377 articles. We screened these articles retaining those in which animal DNA samples were actually collected, leading to 349 articles, and removed articles with insufficient methodological information to conclude on the specific questions investigated. A total of 342 papers were retained in our final dataset (see list in Supplementary Table 1). Although this dataset may not be exhaustive; it is taken to be representative of the current literature on non-invasive DNA sampling.

During the same time period and in the same fields as above, we estimated the total number of articles focusing on invertebrates versus vertebrates using the following commands:

- (TS=(mammal) OR TS=(vertebrate) OR TS=(bird) OR TS=(amphibian) OR TS=(reptile) OR TS=(fish) NOT (TS=(insect) OR TS=(invertebrate) OR TS=(crustaceans) OR TS=(annelid) OR TS=(echinoderm) OR TS=(nemathelminth) OR TS=(arachnids) OR TS=(arthropod) OR TS=(plathelminth)) AND SU=(ecology OR zoology OR ornithology OR ecology OR environmental sciences OR entomology OR fisheries OR behavioural science OR Biodiversity & Conservation) AND PY=(2013 OR 2015 OR 2017 OR 2014 OR 2016 OR 2018))
- (TS=(insect) OR TS=(invertebrate) OR TS=(crustaceans) OR TS=(annelid) OR TS=(echinoderm) OR TS=(nemathelminth) OR TS=(arachnids) OR TS=(arthropod) OR TS=(plathelminth) NOT (TS=(mammal) OR TS=(vertebrate) OR TS=(bird) OR TS=(amphibian) OR TS=(reptile) OR TS=(fish)) AND SU=(ecology OR zoology OR ornithology OR ecology OR environmental sciences OR entomology OR fisheries OR behavioural science OR Biodiversity & Conservation) AND PY=(2013 OR 2015 OR 2017 OR 2014 OR 2016 OR 2018))

The results from these searches were used as non-exhaustive but comparable numeric estimates only, and were therefore not further curated. The abstract and method section of each of the papers were carefully screened to check whether the methods used complied with the original definition proposed by Taberlet et al.[6] or not. A middle-ground category, labelled as “potentially affecting territory”, was created for cases where faecal samples were taken from wild animals that are known to use dejections as territory or social marking. We excluded from this category, studies that specifically mentioned only partial collection of faeces. Where multiple methods were used in the same study, these were classified as compliant with the definition by Taberlet et al. only if all the methods used were compliant or if invasive sampling methods were clearly identified from non-invasive ones. The latter required screening of the whole paper.

Statistical analyses were conducted with R[7] (version 3.6) and RStudio[8] (version 1.2.1335). Packages used included *stats*, *googleVis* and *bipartite*. Statistical significance was set at 5%.

3. WHAT NON-INVASIVE DNA SAMPLING IS USED FOR

Our systematic review captured 342 articles for which samples were collected from 92 different countries on all continents except Antarctica (Fig 1a). The number of papers detected per year was stable between 2013 and 2017 ($X^2 = 1.4045$, df = 4, p-value = 0.8434). Similarly, there is no significant variation in the proportion of the different methods used between 2013 and 2017 ($X^2 = 20.243$, df = 20, p-value = 0.4429) (Fig 1b).

Among the studies captured in our review, 43% aimed at identifying organisms at the species level, for example to produce biodiversity inventories, or at the individual level (Fig 1c). The latter was often conducted in the context of Capture Marking Recapture (CMR) studies (e.g.[9]), where it is essential to identify individuals. Individual genotyping was also often attempted to measure genetic diversity or for population genetic studies (e.g.[10]) in 14% of the reviewed articles. The development of new protocols where the quality of the DNA obtained non-invasively was the centre of interest was the aim in another 14% of the studies. Other recurrent foci were on the detection of presence (11%), the study of animals' diet (7%) or the sexing of individuals (6%).

The type of samples collected varied widely and 25 different categorised were recorded. However, a large number of the studies focused on faecal samples collected as eDNA (47%) (Fig 1c). Another 20% of studies were based on the collection of more than one type of samples, often including faeces. Hair samples, water samples and feathers were the following most represented sample types in our dataset (11%, 4% and 3% of studies respectively). Hair samples were mainly collected through DNA trapping, while feather and water samples were generally collected using an eDNA approach. We also uncovered a variety of much more atypical sample types such as insect pupal cases, urine, fingernails, placenta, mucus etc.

Overall, the substantial majority of sampling methods (72%), were based on the collection of eDNA, while DNA trapping was rarely used (11%). Other cases included studies using several different methods (12%) and few very specific cases (Fig 1c). For example, invertebrates such as leeches[10] and carrion flies[9] were used to sample the DNA of the species on which they feed (Fig 1c). More surprising was the presence of non-lethal and lethal sampling methods applied to so-called non-invasive DNA sampling (9% of the reviewed papers). Such methods are in breach of the definition of non-invasive DNA sampling as proposed by Taberlet et al.[6]. In fact, 59% of reviewed papers using the phrase "non-invasive" or "noninvasive" did not comply with this definition (Fig 2a) even when this phrase was present in the title of the article (60% of non-complying articles).

4. DNA COLLECTION AND THE NON-INVASIVE MISNOMER

Subsequent to its original definition, the term non-invasive has often been misapplied in the literature [11]. In practice, so-called 'non-invasive' methods have often encompassed DNA collection techniques that preserve the physical integrity of an organism but have an unmeasured, and potentially significant, impact on the fitness, behaviour or welfare of the subject being studied. For example, the following DNA collection methods were all defined as 'non-invasive' by the respective authors: gentle pressure applied to the thorax and abdomen of carabid beetles (*Poecilus cupreus*) to trigger regurgitation [12]; flushing of sage-grouse (*Centrocercus urophasianus)* from their roost sites to collect fresh faecal pellets [13]; and trapping, handling and cloacal swabbing of lizards (*Phrynosoma cornutum*) [14]. Misleading use of terminology in biology and ecology is a longstanding concern [15–17]. To demonstrate the extent of the issue, we conducted a systematic review of the recent literature (2013-2018) and evaluated how well papers using the term "non-invasive DNA sampling" complied with the original definition by Taberlet et al. [6].

When non-invasive DNA sampling is misapplied, readers unfamiliar with the scientific literature on DNA sampling (e.g. decision makers, conservation managers, and other end-users), may be misled in thinking that the described method can be applied without affecting the fitness nor behaviour of the target animals. Misnaming DNA sampling is also problematic for assessing impact on animals, identifying opportunities for refinement, and for ensuring validity and quality of the data collected. Using more precise terminology could also help scientists realise that they may have been using invasive methods after all, and encourage them to consider reducing the impact of their sampling and/or search for truly non-invasive alternatives. The main issues exposed by our literature search are summarised in Box 1.

Box 1: THE SEVEN SINS OF NON-INVASIVE DNA SAMPLING

Sin 1: Taxonomic bias

One conspicuous result from our review was that only 18 studies (~5% of the reviewed papers) focused on invertebrates compared to 321 focusing on vertebrates (Fig 2b). This striking imbalance implies that non-invasive methods are rarely considered for sampling invertebrate DNA. Even when authors claimed to use non-invasive DNA sampling on invertebrates, they used methods that alter the physical integrity of the organism in 40% of the cases (Fig 2d). For example, Rorat et al. [18] collected individual earthworms, which they then electrified "*lightly*" to induce coelomic secretion. Yet, truly non-invasive methods exist for invertebrates, for example through field collection of insect exuviae [19], pupal cases [20], empty mummies [21], dust [22] , soil[23], or water samples[24].

The use of non-invasive DNA sampling and the misuse of the term also varies in relation to the taxonomic group of interest within vertebrates (Fig 2c) (X^2 = 165.17, df = 30, p < 2.2e-16). For example, 43% of the studies on fish involved alteration of the physical integrity of the organism. These included fin clipping in eels (*Anguilla anguilla*) [25] and sting amputation in rays (*Aetobatus narinari*) [26] which were both considered non-invasive because these body parts can regenerate, despite the fact that fin clipping is known to be painful for fish [27].In comparison, only 3% of the studies focusing on mammals, involved biopsies.

Sin 2: Misclassification of faeces as non-invasive DNA samples

The majority of the literature on non-invasive DNA sampling included the collection of faecal samples (58% of all studies reviewed here). Faecal collection is very prevalent in the field and assumed to be non-invasive by most authors. However, our analysis shows that 46 % of the studies focusing solely on faecal sampling did not comply with the original definition of non-invasive DNA sampling. This included detection of animals and collection of faecal samples using aircraft (e.g. [28]), which may increase stress in animals (e.g. [29]) or cases where animals were being held in captivity (e.g. [30]), specifically captured to obtain faecal samples (e.g.[31]), or even killed prior to sample collections[32]. For example, 17 of the faecal samples analysed by Kolodziej et al.[32] were obtained from the rectum of feral pigs (*Sus scrofa*) that had been hunted. Similarly, Jedlicka et al. [33] "extracted DNA from noninvasive fecal samples" of Western Bluebirds (*Sialia mexicana*) by catching adult and placing them in brown paper bags. Despite focusing on faecal samples, these procedures do not fit the definition proposed by Taberlet et al. [6]. The central misconception, here is that there is no such thing as "non-invasive DNA samples". Rather than the type of sample, it is the method of sampling that needs to be scrutinized for its invasiveness. Another key issue with faecal sampling is that many animals mark their territory using faeces to dissuade potential intruders (e.g. in wolf communities, see [34]) and also use such marks to recognise individuals from neighbouring territories, avoid unnecessary conflict and promote non-agonistic social encounter such as mating. Therefore, even when collected opportunistically after the animal has left, faecal sampling can in some cases affect the marking behaviour of territorial species (e.g. [35]) (Fig 2a). Such effect will likely vary with the ecology of the taxa studied but can be particularly significant for small animals when the entire scat is collected, or if undertaking repeated sampling (e.g. [36]). The collection of samples from territory boundaries must therefore aim to preserve territory delineation and socially relevant information. Unless the species is known to be non-territorial or marks its territory with cues other than those collected (e.g. maned wolves (*Chrysocyon brachyurus*) mark their territories with urine [37]), precautions should be taken to avoid impacts on marking and other social behaviours. These risks could be easily alleviated by only collecting a small portion of a faecal sample. We recorded six studies where this issue was clearly addressed either by swabbing faeces without removal [38] or by only collecting scat subsamples [39–43].

Sin 3: Baiting DNA traps

In most studies using a DNA trapping strategy (91%), researchers employed bait or lures to increase the yield of their traps. Very few studies used non-lured DNA traps, for example, barb wire placed at sites used by brown bears (*Ursus arctos*) [44,45] or modified body snares at otter (*Lontra canadensislatin*) latrine sites, to collect hair [46]. Although it seems perfectly legitimate (and often essential) to increase the attractiveness of DNA traps with food [47], scent marks from other individuals [48] or other attractants (e.g. Valerian essence for cats) [49], the animal's behaviour will obviously be disturbed as a consequence and therefore, these methods cannot be considered fully non-invasive sensu Taberlet et al. [6].

Sin 4: Combining invasive and non-invasive methods

In a few examples the impact of the sampling strategy on the animal behaviour is obvious from the article's title itself, for example when baited traps are mentioned (e.g. [49]).

However, in many more papers (n=35) confusion arises because authors used the phrase "non-invasive sampling" or "non-invasive DNA sampling" while a variety of sampling techniques were actually applied, some of which were non-invasive and some of which were invasive sensu Taberlet et al. [6]. This lack of clarity about what is non-invasive and what is not can be misleading for the reader. Some authors clearly stated the invasiveness of the different methods used (e.g. [32,50–52]), however, most papers where mixed DNA sampling strategies were applied did not specify which of these methods were considered non-invasive.

Another facet of this issue arises when tools (e.g. new primers, extraction protocols, DNA conservation methods) are developed specifically for analysing samples collected non-invasively but are actually tested only (or partly) on samples that were collected invasively (n=18) for example by capturing animals to perform the sampling (e.g. [53,54]). It is essential in such cases that authors fully acknowledge the invasiveness of the sampling method they used. Often this is not clearly specified.

Sin 5: A bird in the hand is no better than two in the bush

Trapping and restraint of wild animals is recognised as a significant stressor that can result in distress, injury, and death (e.g. [55]). Capturing and/or handling animals for DNA sampling was observed in 25% of all articles reviewed here (Fig 2c), despite the clear definition given by Taberlet et al. [6] that non-invasive DNA is "*collected without having to catch or disturb the animal*". Indeed, capture and/or handling of individuals to obtain DNA samples (e.g. saliva swabbing) can induce long-lasting stress effects [56,57], and there are very few cases where capturing an animal might have no effects on its future behaviour. Therefore, when animals must be held captive, transported or restrained in order to perform DNA sampling, the method cannot meet the definition of non-invasive DNA sampling *sensu stricto* [6]. Skin swabbing of octopus (*Enteroctopus dofleini*) for example [58], is unlikely to be possible in the wild without disturbing the animal and the potential negative impacts on animal welfare (see [59] for a review on cephalopod welfare) must still be recognised.

Another common scenario where the animals are held during DNA sampling relates to the use of museum specimens or animals that were killed for other purposes (n=4). Whether they were legally hunted or poached and confiscated (e.g.[60]), this type of sampling does not qualify as non-invasive due to the disturbance and/or death of the animal through human activity. Often, a better term for such sampling is "non-destructive", which does not damage the specimen [61,62] (Table 1). On the other hand, tissue sampling from animals that were found dead of natural causes is analogous to eDNA left behind by a free ranging animal and can be considered non-invasive (e.g. [63]). It should be noted, however, that opportunistic sampling from animals already killed for other purposes (e.g. culling, museum samples) may be an ethical option because it reduces the need to otherwise target living animals and conforms to the principle of Reduction (reducing the number of affected animals) under the 3Rs framework.

Sin 6: All or nothing

Only 41% of the reviewed studies fully met the criteria of the original definition of non-invasive DNA sampling. In most cases, however, authors tried to minimise the impact of sampling, but the nature of the definition proposed by Taberlet et al. [6] leaves no middle ground between invasive and non-invasive sampling methods. One potential solution to this is to use the term "minimally-invasive DNA sampling", which can be defined as obtaining DNA with minimised effects on the animal's structural/physical integrity, and potential impact on the behaviour and welfare of the organism (Table 1). In our dataset, this term was used in six studies to qualify skin swabbing of fish [64], amphibians [65] and bats [66], feather plucking of gulls [67], cloacal swabbing in rattlesnakes [68] and ear biopsies in rodents [69]. A broader use of this term would lead to more accurate reporting, for which potential impacts of the sampling are acknowledged, while still emphasising the aspiration of the authors to minimise those impacts. The challenge associated with the use of such a term would be to define where ambiguities fall between minimally-invasive and invasive sampling methods.

Sin 7: Using the common signification of non-invasive sampling

The lack of perceived stress or pain experienced by an animal is often used as a criterion to support the classification of a method as non-invasive. For example, du Toit et al. [70] stated that "*Pangolin scales consist of non-living keratin, therefore taking scale clippings is considered to be non-invasive*". This statement relates to the common definition of a "non-invasive" medical or veterinary procedure, i.e. one that does not involve puncture of the skin or other entry into the body [71]. This definition (rather than the one by Taberlet et al. [6]) seems to be the one adopted by most authors (93% of the reviewed papers complying) (Fig 2d). This was also the case for several articles at the frontier between medical/veterinary fields. Kauffman et al. [72] for example, called the sampling of vaginal swabs and urine from captive dogs non-invasive. Similarly, Reinardy et al. [73] designated as 'non-invasive' a procedure consisting of "*lightly anaesthetizing fish and applying a slight pressure on their abdomen to expel sperm*", which was then used for DNA analysis. These examples were rare in our dataset (n=3) probably because of our strict selection of articles from non-medical and non-veterinary domains (see selected fields in section 2). Nonetheless, as science becomes increasingly transdisciplinary and genetic methods developed in neighbouring fields are used in ecology, this type of confusion is likely to become more prevalent in the future. The discrepancy with the common definition of a non-invasive procedure comprises a significant limitation of the phrase non-invasive DNA sampling as defined by Taberlet et al. [6], and importantly, could minimise the perceived impacts of sampling methods on animal welfare, even if these impacts are significant in reality. Although this issue was first highlighted in 2006 by Garshelis who stated that: "*the term noninvasive has 2 distinct meanings, 1 biological and 1 generic, which have become intertwined in the wildlife literature*" [11], the confusion continues to riddle the current literature.

5. INTRODUCING THE TERMS NON-DISRUPTIVE AND MINIMALLY DISRUPTIVE DNA SAMPLING

In order to clarify some of the existing discrepancies exposed by our literature review, we propose the introduction of the term, 'non-disruptive DNA sampling'. This term emphasises the effects of the sampling method not on the physical integrity/structure, but on the fitness and behaviour of the organism from which the sample is obtained. We define 'non-disruptive DNA sampling' as obtaining DNA from an organism without affecting its fitness, or causing any behaviour or welfare impact that may last longer than the duration of the sampling (Table 1). We define 'minimally disruptive DNA sampling' as any sampling method that minimises impacts on fitness, behaviour and welfare. Non-disruptive DNA sampling can be differentiated from 'non-invasive DNA sampling' which in the current literature, largely focuses on whether the method of sampling impacts physical structures of the animal (Fig 2d). The introduction of 'non-disruptive DNA sampling' terminology provides a functional term that appropriately focuses on the impact to the individual and not on a specific quality of the methodology (e.g. whether a physical structure is altered). We acknowledge that very few current DNA sampling methods may be entirely non-disruptive, and recommend that researchers aim at minimising disruption through protocol refinement. This could be achieved by testing the potential effects of different DNA sampling methods on survival, stress, behaviour and reproduction success as a proxy for fitness. In order to make our intended meaning clear, we overlaid existing DNA sampling terms in relation to non-disruptive DNA sampling methods in the following paragraphs and in Figure 3. Rather than debating and refining existing terms, the essential point of Figure 3 is to distinguish between disruptive methods, which are likely to cause lasting effects on the behaviour, welfare or fitness of an organism, and non-disruptive ones, which may not.

5.1. Impact of DNA sampling on behaviour, fitness and welfare

Studies examining the effect of DNA sampling on behaviour, fitness and welfare are rare and their results are not always predictable. For example, the fitness consequences of DNA sampling methods, often measured using individual survival as a proxy for fitness (e.g. [74–76]), depends on the taxa sampled. Responses may vary strongly between species [77] and even between males and females of the same species. For instance, Vila *et al.* [78] showed that the non-lethal but invasive DNA sampling through leg or hind wing clipping had an effect on survivorship and reproductive behaviour of adult males of the protected moth *Graellsia isabelae,* while mid leg clipping had a negative impact on female mating success. In particular cases, procedures to obtain DNA samples can also increase the fitness of animals. For example, supplementary feeding can have a direct positive impact on the fitness of birds [79], and this may occur when animals are attracted to DNA traps baited with food or feeding cages where animals are caught for DNA sampling (e.g. [80]). In mammals, remote DNA sampling using biopsy darts is known to cause little reaction from marine mammals when conducted correctly and is unlikely to produce long-term deleterious effects [81]. Gemmell and Majluf [82] found that in most cases New Zealand fur seals (*Arctocephalus forsteri*) recoiled from the impact and searched briefly for the assailant, but never abandoned their territory following the darting. Another study found that bottlenose dolphins (*Tursiops* spp) reacted similarly to the darting process regardless of being hit or not, suggesting that the reaction is mainly caused by 'unexpected disturbance' rather than biopsy [83]. No sign of long term altered-behaviours was observed, including probability of recapture. Despite this, all biopsy sampling involves some level of risk [81], and different individuals from the same species may react differently to similar stressful situations depending on gender [84] or individual physiological and psychological factors [85,86]. With regards to animal welfare, Paris et al. [87] assessed the impact of different DNA sampling methods on individual welfare in frogs. They concluded that capture and toe clipping was significantly worse than capture and buccal swabbing in terms of the level of suffering experienced by an animal, and the detrimental impacts on survival. These examples illustrate that the level of disruptiveness of DNA sampling methods should be made cautiously and studies assessing their impact on fitness, behaviour and welfare should be encouraged prior to their use.

5.2. Examples of non-disruptive or minimally disruptive DNA sampling

Non-disruptive DNA sampling comprises all non-invasive DNA sampling *sensu stricto* i.e when the DNA is collected without the subjects being aware of the researcher's presence or experiencing any detrimental effects (as suggested in Taberlet & Luikart [5]). For example, most eDNA sampling and DNA trapping methods do not require researcher and subject to be present at the same time and place. An important point of difference between these two methods is that eDNA is often collected somewhat opportunistically, while DNA trapping allows for strategic spatial distribution of sampling.

Examples of DNA trapping that are non-disruptive include remote plucking or hair trapping by means of unbaited hair snag traps [88,89] or tape [90,91] placed at well-used runs. Environmental DNA sampling includes field collection of faeces (e.g. [37]) as long as these do not affect territory marking (see section 3.2), DNA collection from footprints in the snow, such as those from the Swedish Arctic fox (*Vulpes lagopus*) [92], and from saliva on twigs, such as from ungulate browsing [93]. When DNA is collected in the presence of the animal, the effects of sampling can be minimised by avoiding or drastically limiting handling. For example, the swabbing of animals directly in the field with little [94] or no handling [95].

Sampling methods that are non-disruptive have many benefits for wildlife conservation, because they are unlikely to introduce bias or experimental effect or impact on animal welfare. However, they may be limited in their applicability. The main limitations associated with eDNA and DNA trapping include low DNA quantity and quality [96], as well as potential contamination from non-target species [97]. Another limitation of DNA trapping might be the mixture of DNA from several different target individuals. In such instances, next-generation sequencing (NGS) or other post-PCR analysis (e.g. cloning, single stranded conformation polymorphism, high resolution melting, denaturing gradient gel electrophoresis) might be required to differentiate and identify the DNA of each individual.

A shift in focus from sampling methods that aim at avoiding breaches to physical structures of an organism, to non-disruptive or minimally disruptive methods, (avoiding impact on behaviour, fitness or welfare), means in some cases the most appropriate method may be invasive but results in a lower impact on the animal. For example, invertebrate antenna clipping in the natural environment breaches a physical structure but may result in no effect on survival (e.g. [76]) and may have lower impacts than collecting and removing specimen to captivity for faecal sampling or forced regurgitation.

Similarly, remote dart biopsy or flipper notching of marine mammals are often a preferred choice over stressful captures for DNA sampling because they only cause short term effect (if any) on the behaviour of the animal [98,99]. Under our definitions, hair collection from the environment, unbaited DNA traps, skin swabbing in the field or remote darting on wild sea mammals could be considered non- or minimally disruptive (Fig 3).

6. WHEN IS NON-DISRUPTIVE DNA REQUIRED OR PREFERRED?

Non-disruptive DNA sampling provides a compromise between minimising welfare and ethical costs, and obtaining a quality DNA sample. DNA sampling methods where the specimen is in hand generally results in fresher and better-quality DNA, despite the potentially higher impact on animal behaviour or welfare. While the welfare of all experimental animals should be considered, when the subject is endangered or afforded legal protections there may be additional welfare and/or ethical issues surrounding the use of invasive DNA sampling techniques [74,100]. Additionally, the test subject may be required to be alive for further testing or return to their natural habitat. If further tests involve capturing an animal for a laboratory experiment [101] or for translocation [102], then the effects of capturing and holding the organisms for DNA sampling are of less concern as individuals will need to be captured for these experiments anyway. However, stressful events can have a cumulative effect [103] therefore the potential further exacerbation of stress by DNA sampling should be carefully considered.

The importance of considering non-disruptive DNA sampling also depends on the type of study undertaken. Below we describe experimental studies, field behavioural studies, and capture mark recapture (CMR) research, as three types of situations in which collection and use of non-disruptive DNA samples may be essential.

6.1. Laboratory-based experimentations

Non-disruptive DNA sampling is necessary for species identification, sexing or genotyping of individuals prior to laboratory-based experimentation where fitness and/or behavioural traits are to be assessed. For example, many species of birds are monomorphic, and can only be sexed using molecular analysis [104]. Similarly, many cryptic species complexes can only be elucidated genetically [105]. Laboratory-based behavioural or fitness studies involving cryptic or monomorphic species may therefore require DNA sexing or species identification of individuals before conducting research on them [101,106] to ensure a balance of sex or species across different treatments. Even when species identification is not an issue, the organisms being studied may comprise different morphocryptic genotypes [106] that need to be determined prior to experimentation in a way that does not affect their fitness or behaviour. One classical way to alleviate the effects of sampling on behaviour (for example when animals are collected in the wild and brought to the lab), is to allow for a recovery and acclimation period.

6.2. Behavioural studies in the field

The second major use of non-disruptive DNA sampling is when relatedness between individual subjects needs to be determined prior to a behavioural study conducted in the field. For example, social interactions in mammals are often linked to kinship and can be mediated by the physiological state of individuals [107]. The capture and handling of animals can modify their physiology [108], thereby affecting their social behaviour. Recent studies also suggest that although behaviours observed shortly after release may appear 'normal', stress levels may still be high and impact activity budgets [109]. Such effects may remain undetected but have significant implications for subsequent data reliability and validity.

6.3. Capture Mark Recapture

The effects of DNA sampling on animal behaviour may also affect the results of studies that are not directly examining behaviour or fitness. The third case when non-disruptive DNA sampling is recommended is when doing Capture Mark Recapture (CMR) studies. CMR studies using DNA tagging are often conducted to estimate population size (e.g. [110]), with the additional benefit of enabling population genetic analysis on the samples collected. Invasive or disruptive DNA sampling techniques may affect the survival rate of marked individuals, or introduce avoidance behaviours, which may cause trap avoidance, and the population size to be overestimated. For example, toe clipping combined with CMR is commonly used to estimate population abundance of amphibians [111], but toe clipping has been shown to decrease chances of frog recapture by 4 to 11 % for each toe removed [74]. Similarly, sampling methods that may increase the fitness of animals (e.g. feeding cages or baited DNA traps) could lead to previously sampled animals to be more attracted than naïve ones (Boulanger et al. 2004, Gashelis 2006), thereby biasing the CMR results towards underestimating population size.

Such biases can be limited by the use of non-disruptive DNA sampling methods. Although eDNA has been used in CMR studies and is in most cases non-disruptive, it can have some limitations. The presence of mixed DNA samples and the lower quality of the collected DNA can lead to false positives where animals not captured previously are believed to be recaptured due to their DNA profile being an indistinguishable shadow of previously captured animals [112]. Because of this, non-disruptive DNA sampling may provide an appropriate balance between sample quality, data quality and impact on animals.

7. TAKE-HOME MESSAGES

1. In practice, most papers using the phrase "non-invasive DNA sampling" only comply to the medical definition of the term non-invasive, which is broader than the original definition proposed by Taberlet et al. [6] and is concerned only with the preservation of the physical integrity of the organism being sampled. We urge scientists using non-invasive DNA sampling methods to always state whether they refer to the definition by Taberlet et al. [6] *sensu stricto* or the medical definition of a non-invasive procedure (*sensu lato*).
2. We propose the new terms, "non-disruptive" and "minimally-disruptive" DNA sampling, to more appropriately address the potential behaviour, welfare and/or fitness effects of DNA sampling methods, as opposed to physical integrity (invasiveness in the medical sense). We can envisage situations in which the research aims are not impacted by the sampling approach to obtaining DNA. However, researchers have an ethical obligation to minimise the impacts on the animals. Therefore, whenever possible, non-disruptive or minimally disruptive DNA sampling methods should be selected, in particular prior to experimental or observational studies measuring fitness or behaviour, as well as studies using techniques such as CMR where fitness or behaviour may affect results.
3. It may in some cases be better to use a physically invasive method (e.g. remote biopsy) that is minimally disruptive rather than a method that does not involve puncturing the skin but causes severe stress and has long-lasting effects (e.g. stressful capture for saliva swabbing).
4. More research is required to better understand the consequences of different live DNA sampling methods on behaviour, welfare and fitness in a variety of animal species and contexts.

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AUTHOR CONTRIBUTIONS

Conceptualised the idea: MCL, SB, RHC ; recruited co-authors and organised literature review and writing workshops: MCL, SB ; conducted the systematic review: MCL, SB ; prepared the figures: SB ; drafted and revised the manuscript MCL, RHC, NA, AB, KD, AK, JR, VRS, RS, WB, SB. The overall author percentage contributions are as follow: MCL[25], RHC[10], KD[8], NA[5], AB[5], AK[5], JR[5], VRS[5], RS[5], BW[2], SB[25].

DATA AVAILABILITY

The list of publications reviewed and the raw data used for the analyses are available in Supplementary Table 1.

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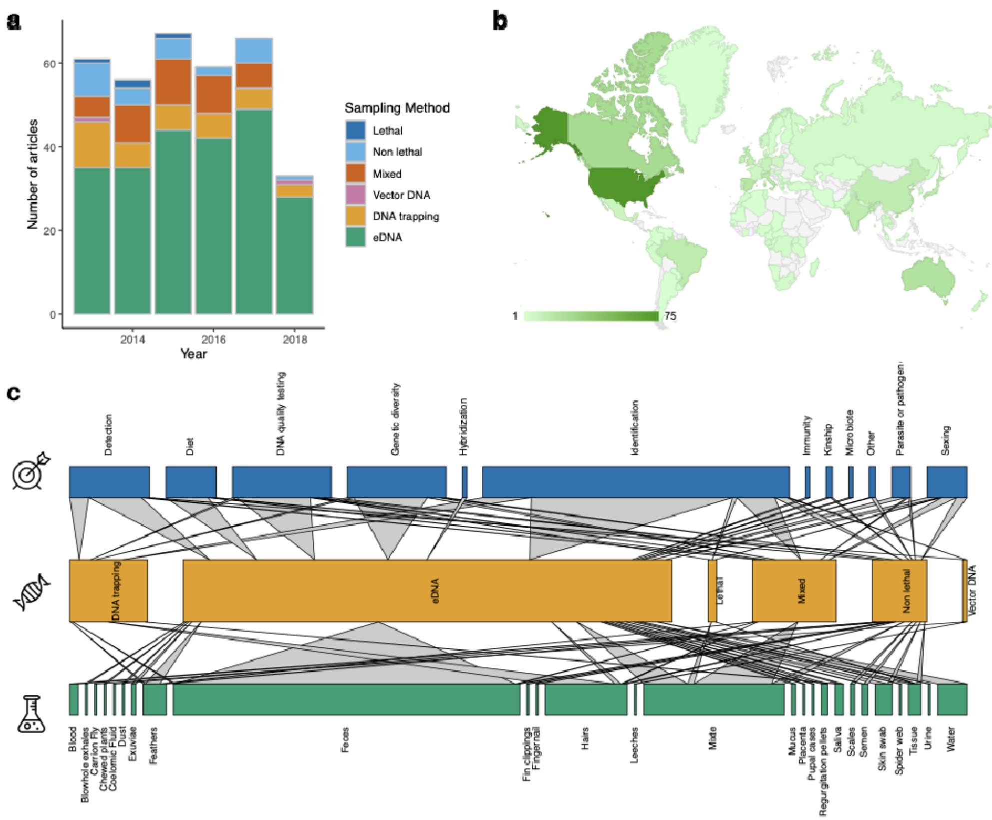

Figure 1. Summary statistics of the literature review on the use of "non-invasive DNA sampling" between January 2013 and May 2018 (n=342).

a: Number of articles in relation to the sampling method used between 2013 and 2018. The year 2018 is incomplete as the search was performed in July 2018.

b: Countries of origin of the samples analysed in the reviewed papers. Countries in grey were not represented in our review, countries coloured in various shades of green provided samples for 1 to 75 of the reviewed papers (see in-graph legend for colour scale).

c: Bipartite network of the main aim of the studies in blue, the type of sampling method used in orange (see Table 1 for definitions) and the nature of the samples collected in green. The horizontal width of the rectangles represents the number of articles in each category.

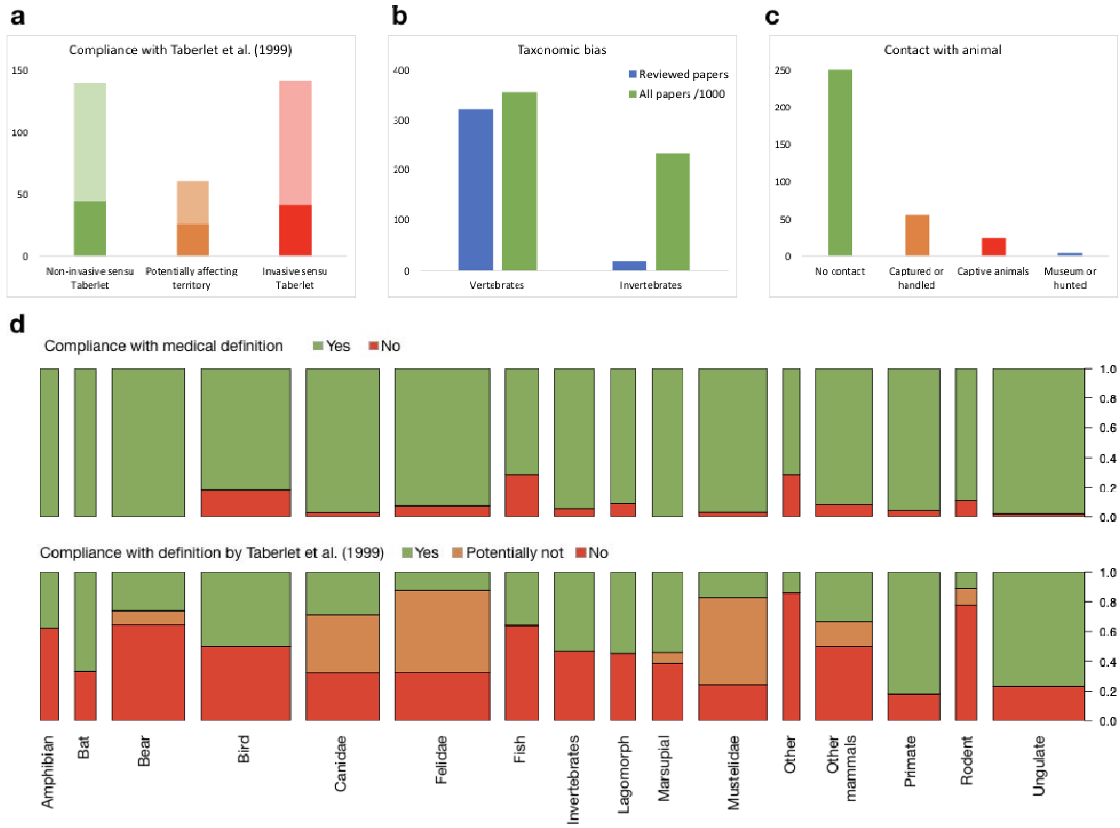

Figure 2. Summary statistics of the main issues exposed by our literature review on the use of "non-invasive DNA sampling" between January 2013 and May 2018 (n=342). For a, b, and c, the y-axis is number of papers. For d, the y-axis is the proportion of papers and the width of the bars represents the number of papers for each taxonomic group.

a: Compliance of papers with the original definition proposed by Taberlet et al. ([6]). Studies where multiple methods were used (n=31) were classified as compliant with the definition by Taberlet et al. only if all the methods used were compliant OR if invasive sampling methods were clearly identified by the authors. Dark colours correspond to papers where the phrase "non-invasive" was present in the title, lighter colours correspond to papers where the phrase "non-invasive" was not present in the title. The orange bar (labelled as "potentially affecting territory", corresponds to cases where territory marking and social interactions may have been affected by the removal of faecal samples.

b: Taxonomic bias in the non-invasive DNA sampling literature. Number of papers reviewed that focus on invertebrates or vertebrates compared to all papers on invertebrate or vertebrate (see Method section for search command).

c: Number of papers complying (in green) or not complying with the no contact criteria proposed by Taberlet et al. ([6]), because animals were either captured or handled for DNA sampling (orange), held in captivity (red) or had been killed (blue). **d**: Proportion of papers complying with different definitions of non-invasive sampling in relation to the taxonomic group studied. Top: compliance with the common definition of a non-invasive medical or veterinary procedure, (i.e. one not involving puncture of the skin or other entry into the body ([71]). Bottom: compliance with the definition of non-invasive DNA sampling proposed by Taberlet et al. ([6]). Orange boxes (labelled as "Potentially Not") correspond to cases where territory marking and social interactions may have been affected by the removal of faecal samples.

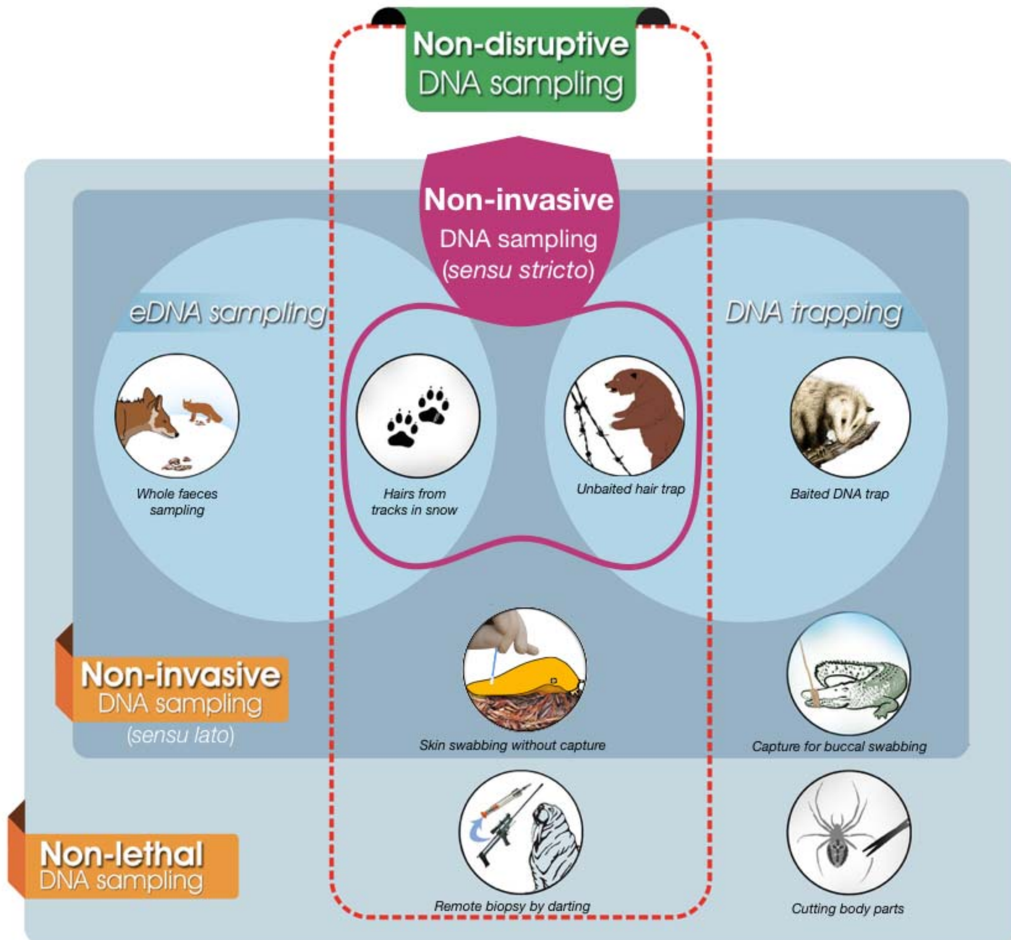

Figure 3. The relationship between non-disruptive, non-invasive and non-lethal DNA sampling methods. Non-invasive DNA sampling *sensu stricto* corresponds to the definition given by Taberlet et al. ([6]), Non-invasive DNA sampling *sensu lato* corresponds to the medical definition ([71]). Pictograms represent a non-exhaustive list of examples for which references are given below. From left to right and top to bottom: whole faeces sampling for species that use faecal territory marking ([113]), hairs collected in snow ([51]), hairs collected with unbaited barbed wire([44]), DNA trap baited to attract animals ([114]), skin swabbing in the field without capture ([95]), capture of reptiles for buccal swabbing ([115]), gun darting of big mammals to collect tissue sample([116]), biopsy on handled invertebrate ([117]).

916 **Table 1. Glossary of terms as used in this review.**

Term	Definition
DNA trapping	Remotely obtaining DNA from one or more unknown individual organisms by taking a sample while they are present. This usually involves some sort of trap or device, which may or may not be disruptive.
eDNA sampling	Obtaining trace DNA left behind by one or more unknown organisms, by sampling the environment when those organisms are no longer present at the point of sampling.
Minimally disruptive DNA sampling	Obtaining DNA with minimised effects on the animal's fitness, behaviour and welfare. To a minimised extent, such method may affect the structural/physical integrity of the organism.
Minimally invasive DNA sampling	Obtaining DNA with minimised effects on the animal's structural/physical integrity. To a minimised extent, such method may affect the behaviour and welfare of the organism.
Non-destructive DNA sampling	Obtaining DNA from a known individual organism in such a way that the organism may be killed, but not destroyed, so that it can be preserved as a voucher specimen.
Non-disruptive DNA sampling	Obtaining DNA without affecting the animal's fitness, behaviour and welfare.
Non-invasive DNA sampling *sensu lato*	Obtaining DNA without affecting the physical integrity of the animal's through puncturing the skin or other entry into the body (derived from the medical definition of a non-invasive procedure).
Non-invasive DNA sampling *sensu stricto*	Obtaining DNA that was left behind by the animal and can be collected without having to catch or disturb the animal (from Taberlet et al. 1999)
Non-invasive procedure	A procedure that does not involve puncture of the skin or other entry into the body (such as use of an endoscopic device).

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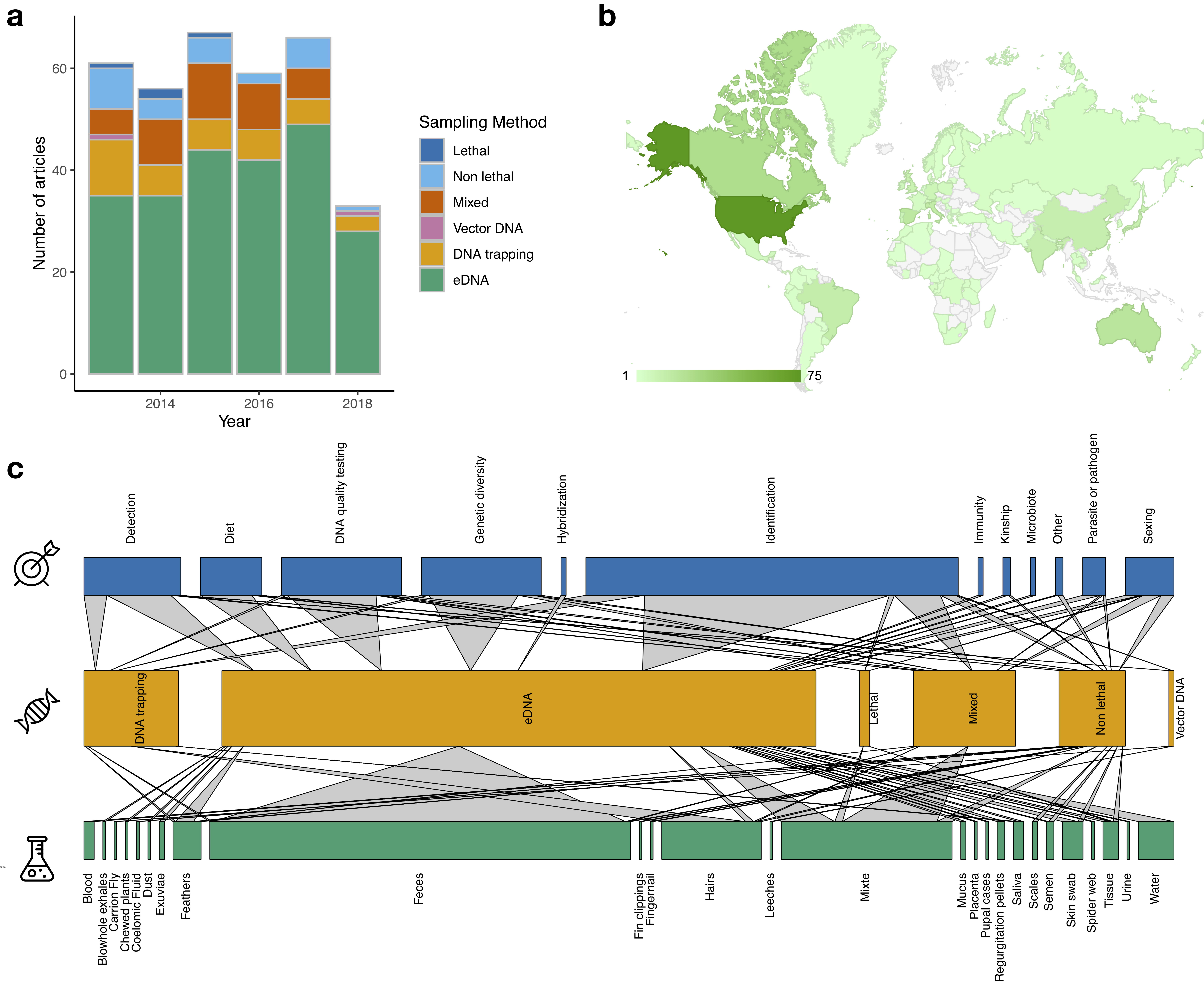
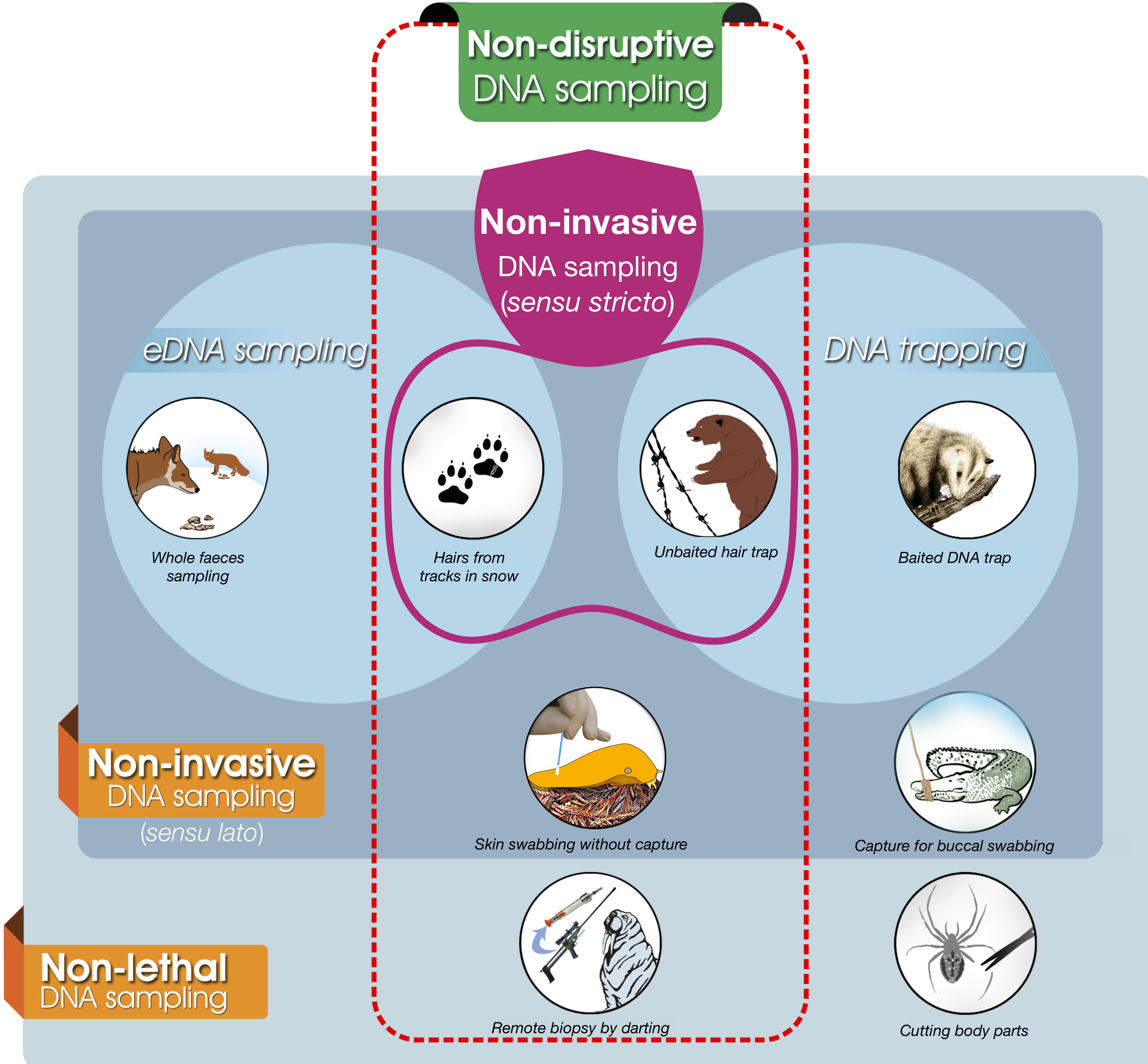

a
Number of articles
0
20
40
60
Year
2014
2016
2018
Sampling Method
Lethal
Non lethal
Mixed
Vector DNA
DNA trapping
eDNA
b
1
75
c
Detection
Diet
DNA quality testing
Genetic diversity
Hybridization
Identification
Immunity
Kinship
Microbiote
Other
Parasite or pathogen
Sexing
DNA trapping
eDNA
Lethal
Mixed
Non lethal
Vector DNA
Blood
Blowhole exhales
Carrion Fly
Chewed plants
Coelomic Fluid
Dust
Exuviae
Feathers
Feces
Fin clippings
Fingernail
Hairs
Leeches
Mixte
Mucus
Placenta
Pupal cases
Regurgitation pellets
Saliva
Scales
Semen
Skin swab
Spider web
Tissue
Urine
Water


a
Compliance with Taberlet et al. (1999)
150
100
50
0
Non-invasive sensu Taberlet
Potentially affecting territory
Invasive sensu Taberlet
b
Taxonomic bias
Reviewed papers
All papers /1000
400
300
200
100
0
Vertebrates
Invertebrates
c
Contact with animal
250
200
150
100
50
0
No contact
Captured or handled
Captive animals
Museum or hunted
d
Compliance with medical definition
Yes
No
Compliance with definition by Taberlet et al. (1999)
Yes
Potentially not
No
1.0
0.8
0.6
0.4
0.2
0.0
Amphibian
Bat
Bear
Bird
Canidae
Felidae
Fish
Invertebrates
Lagomorph
Marsupial
Mustelidae
Other
Other mammals
Primate
Rodent
Ungulate



Non-disruptive
DNA sampling
Non-invasive
DNA sampling
(sensu stricto)
eDNA sampling
DNA trapping
Whole faeces sampling
Hairs from tracks in snow
Unbaited hair trap
Baited DNA trap
Non-invasive
DNA sampling
(sensu lato)
Skin swabbing without capture
Capture for buccal swabbing
Non-lethal
DNA sampling
Remote biopsy by darting
Cutting body parts