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Blood, sweat and tears: non-invasive vs. non-disruptive DNA sampling for experimental biology

Keywords: DNA trapping, eDNA, nonlethal, animal behaviour, fitness, Capture Mark Recapture.

ABSTRACT

DNA data are becoming increasingly important in experimental biology. For example, it may be necessary to obtain DNA from an organism before using it in a bioassay or an experiment, to identify and distinguish between cryptic species, or when comparing different morphocryptic genotypes. Another example could be the assessment of relatedness between organisms prior to a behavioural study. In such cases, DNA must be obtained without affecting the fitness or behaviour of the subject being tested, as this could bias the results of the experiment. This points out the existence of a gap in the current molecular and experimental biology terminology, for which we propose the use of the term non-disruptive DNA sampling, specifically addressing behaviour and/or fitness, rather than simply physical integrity (invasiveness). We refer to these methods as "non-disruptive", and discuss when they are appropriate to use.

SCOPE OF THE PAPER, THE NEED FOR A NEW TERM

With the rise in popularity of molecular methods that has come from progressively cheaper and more user-friendly ways of accessing genomic information (Sboner et al. 2011), DNA data are becoming increasingly important in experimental biology (Suarez and Moyes, 2012). 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 taxa. When DNA analysis is performed prior to experimentation for purposes such as sexing and discrimination between cryptic species, it becomes extremely important to obtain DNA without affecting the fitness or behaviour of the subject being tested, as this could bias the results of the experiment.

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While non-invasive methods of DNA collection focus on preserving the physical integrity of an organism (Baumgardt et al., 2013; Brzeski et al., 2013; Miotto et al., 2014; Waldner and Traugott, 2012), the fitness or behaviour of the subject may still be affected. This uncovers the existence of a gap in the current molecular and experimental biology terminology, specifically addressing behaviour and/or fitness, rather than simply physical integrity (invasiveness).

Non-invasive methods of collecting DNA are increasingly diverse, leading to confusion and misapplication of the term “non-invasive” in the literature . Misleading use of terminology in biology and ecology is a longstanding concern (Murphy & Noon 1991), and the phrase "non-invasive DNA sampling" is no exception. As with many other terms in biology (Herrando Pérez 2014a,b; Hodges 2008, 2014), it has been used in many different and inconsistent ways by various authors. For example, Berry et al. (2013) described hair trapping as non-invasive, requiring no contact between researchers and subjects, while Williams et al. (2012) also described their method of trapping, handling and swabbing the cloacae of lizards as non-invasive. With the aim of clarifying some of the existing discrepancies, we propose the introduction of a new term, “non-disruptive DNA sampling” (Table 1), that emphasises the effects of the sampling method not only on the physical integrity, but also on the fitness and behaviour of the organism from which the sample is obtained.

In order to make our intended meaning clear, we established a list of definitions for the terms used in this paper (Table 1) and the way they relate to one another (Figure 1). Rather than debating and refining existing terms, the essential point of Table 1 and Figure 1 is to distinguish between disruptive methods, which affect the fitness and/or behaviour of an organism, and non-disruptive ones, which do not.

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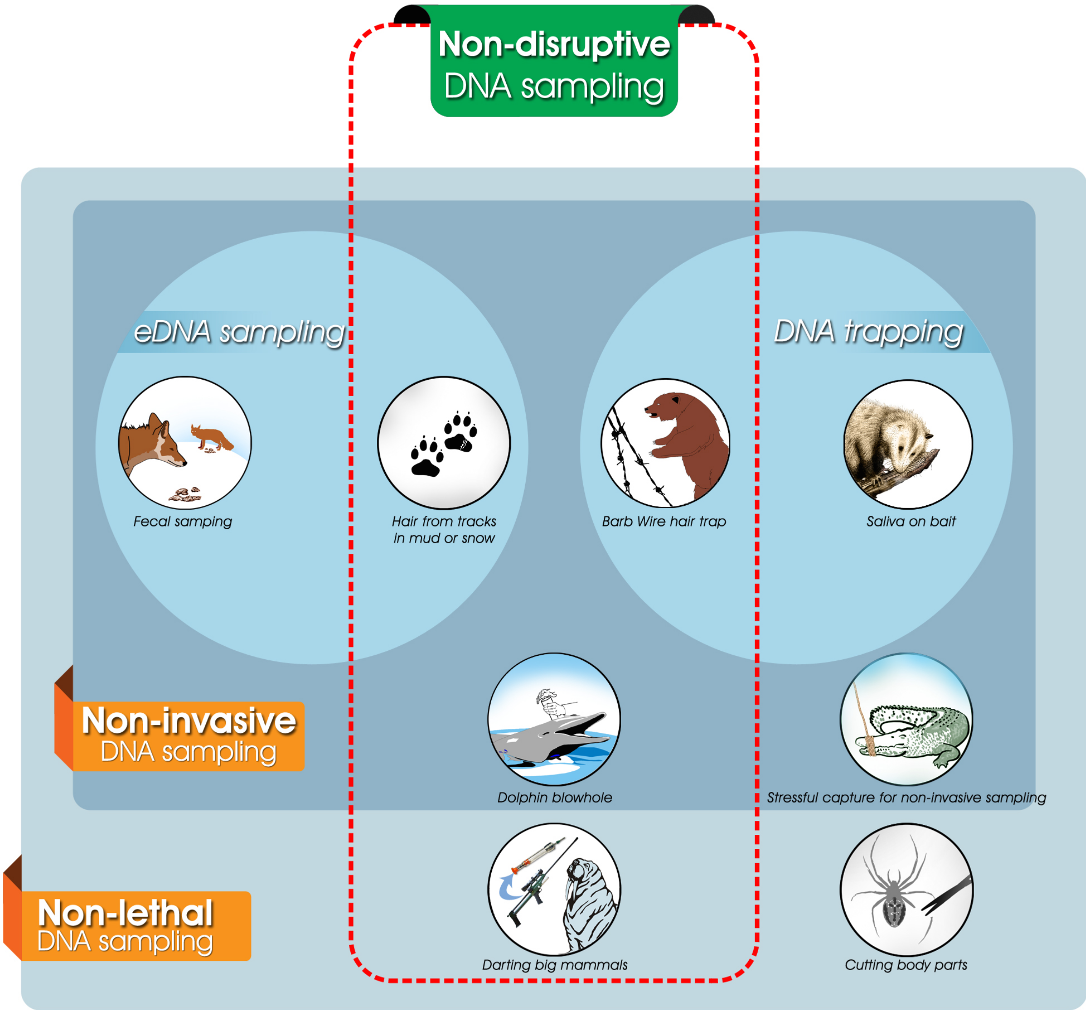

Figure 1. Non-disruptive DNA sampling methods, and their overlaps with non-invasive and non-lethal sampling methods.

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Table 1. Non-exhaustive list of terms, and their definition inherent to the qualification of DNA sampling.

Term	Definition used in this paper	Example
Environmental DNA (eDNA) sampling	Trace DNA obtained from one or more unknown organisms from the environment, when those organisms are no longer present.	Wilcox et al. 2013: sampling fish DNA from stream water
Noninvasive DNA sampling	Obtaining DNA using any method that does not affect the physical integrity of the organism (this includes eDNA; see above), but may affect fitness or behaviour.	Cloaca swabbing of lizards which does not physically harm them, but may affect their behaviour (Williams et al. 2012).
Nondisruptive DNA sampling	Obtaining DNA from a known individual organism without affecting its fitness or behaviour, but may affect the structural integrity of the organism (this differs from some (most) definitions of noninvasive DNA in the literature); this includes DNA trapping (see below).	Removing small parts of the butterfly hindwing does not affect survival or behaviour (Hamm et al. 2010).
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.	Remote hair plucking of badgers with barb wire traps (Frantz et al. 2004)
Noninjurious DNA sampling	Obtaining DNA from one or more known organisms through direct contact, but causing no physical injury or wound. This method may affect behaviour due to minor disturbance.	Skin swabbing of dolphins (Harlin et al. 1999).
Nonlethal DNA sampling	Obtaining DNA from a known individual organism in such a way that its fitness or behaviour are affected but it is not killed (we consider this to be inappropriate if these individuals will be used for experiments following DNA sampling).	Amphibian toe clipping affects survival rate (McCarthy & Parris 2004).
Nondestructive DNA sampling	Obtaining DNA from a known individual organism in such a way that the organism is killed, but not destroyed, so that it can be preserved as a voucher specimen.	DNA extraction from arthropods while keeping them as a voucher specimen (Paquin & Vink 2009).
Intrusive DNA sampling	Obtaining DNA using any method that harms the organism	

NON-INVASIVE CAN BE DISRUPTIVE, NON-DISRUPTIVE CAN BE INVASIVE

Non-invasive DNA sampling methods that are non-disruptive

Many non-invasive DNA sampling methods are de facto non-disruptive because the DNA is collected without the subjects being aware of the researcher's presence, or receiving any detrimental effects. For example, most eDNA sampling and DNA trapping methods do not require researcher and subject to be present at the same time and place. We dissociate eDNA from DNA trapping by the fact that DNA trapping involves the use of a device especially set up for the purpose of DNA sample collection, while eDNA sampling relies on the collection of DNA left behind by animals as they carry out normal behaviours (Table 1). Sampling methods that are both non-invasive and non-disruptive should be regarded as ideal (Figure 1), although they may be limited in their applicability.

An example of DNA trapping that is non-disruptive is remote plucking or hair trapping by means of unbaited barbed wire traps, placed at well-used runs. Remote plucking has been extensively used to collect DNA from mammals and estimate species abundance (Sheehy et al., 2013, Mullins et al. 2010). It is more reliable than direct observation (Frantz et al. 2004) or scat collection (Mullins 2010), and often less time consuming. [DEVELOP EXAMPLES OF eDNA SAMPLING HERE - example of hairs from mud track or snow].

The main limitations associated with eDNA and DNA trapping include low DNA quantity e.g. Asiatic Black Bears (Uno et al., 2012), low DNA quality e.g. lowland gorilla hair (Clifford et al. 1999), and contamination from non-target species e.g. Non-target DNA from multiple fish species in storage tanks (Collins et al., 2012) in the case of DNA trapping. Another limitation of DNA trapping might be the mixture of DNA from several different target individuals. This may not be too problematic for animals that are territorial e.g. the Eurasian badger (Frantz et al., 2004), where only eight out of 130 samples were from multiple individuals). However, if traps are to be left for long time period in areas where several target individuals or species may be present, it is probable that mixed DNA (i.e. from multiple individuals) will be collected (Frantz et al. 2004). The latter would require next-generation sequencing (NGS) or other post-PCR analysis (e.g. cloning, single stranded conformation polyphormism, high resolution melting, denaturing gradient gel electrophoresis) to differentiate the DNA of each individuals.

Non-invasive DNA sampling methods that are disruptive

There is a general assumption that if the physical integrity of an organism is not altered or only slightly altered, then the sampling method is non-invasive. However, only few studies have endeavoured to test this assumption. Nevertheless, it has been proven that capturing individuals with the aim of obtaining DNA samples can be extremely stressful, and therefore disruptive, for the animal. Examples include the

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capture of mammals for saliva swabbing [ADD REF], which induces more stress than instantaneous remote dart biopsy in seals (Emami Khoyi, personal observation) or flipper notching.

Other examples of disruptive but non-invasive DNA sampling arose when the territory of the animal is taken into consideration, particularly when considering eDNA. Faecal DNA can be used, for example, to study the feeding behaviour of endangered species and species of conservation interest (Boyer et al., 2013; Deagle et al., 2010). Additionally, faecal DNA has been used for individual species identification (Lefort et al., 2012). Urine recovered in the snow has also been tested as a potential source of DNA for species and individual identification (Valiere and Taberlet 2000). Despite their non-invasive nature, when chosen these eDNA sources could potentially be disruptive. Many animals mark their territory using faeces or urine; sample collection from the territory boundaries must therefore aim to preserve territory delineation. As illustrated on Figure 1, removing a faecal sample from the environment might affect the marking of territorial species. For instance, it has been shown that removing otter faeces can cause other individuals to believe the area has not been claimed [ADD REF].

Non-disruptive DNA sampling methods that are invasive.

In most cases, eDNA and DNA trapping would be preferred over other non-invasive sampling methods. However, non-disruptive methods where the specimen is in hand, although more invasive, could present advantages. For instance, having the specimen in hand is likely to help retrieve fresher and better quality DNA.

The switch of focus from non-invasive methods, which emphasise avoiding breaches to the physical integrity of an organism, to non-disruptive methods, which are more concerned with minimising effects on behaviour and fitness, means that in some cases the most appropriate method will be invasive. For example, invertebrate antenna clipping in the natural environment may be less disruptive than collecting and taking a specimen back to the laboratory for faecal sampling or forced regurgitation [ADD REF]. Under our definitions, these types of procedure would be considered non-

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disruptive, despite being invasive. In such cases, a less disruptive, more invasive method may yield more DNA of higher quality (Add another reference showing that a more invasive sampling method yielded better DNA).

WHEN IS NON-DISRUPTIVE DNA SAMPLING REQUIRED OR PREFERRED?

DNA sampling methods should be chosen according to the research aims

Non-invasive DNA sampling provides a compromise between welfare and ethics, and obtaining a quality DNA sample. In circumstances where the subject is endangered e.g. endangered tarantulas transported illegally for the pet trade (Petersen et al., 2006), there may be welfare issues surrounding the use of invasive DNA sampling techniques (e.g. Mccarthy and Parris, 2004). In other instances, such as when species are afforded legal protections (Boyer et al., 2013), non-invasive DNA sampling techniques may be preferred. Additionally, the test subject may be required to be alive for further testing, or return to their native habitat. If further tests involve capturing an animal for a laboratory experiment or for translocation (Waterhouse et al., 2014), 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, the potentially stressful effects of capture and manipulation should not be further exacerbated by DNA sampling methods. If animals are to be sampled and observed in the wild, or if welfare or conservation is of concern, the sampling technique must depend on how sampling could affect the fitness and behaviour of the subjects.

The disruptiveness of a particular method varies between species. For instance, (Caudron et al., 2007) suggested that the degree of invasiveness in hair sampling and genotyping from hair follicles in pinnipeds and other marine mammals was influenced by three factors: the duration of sampling, the number of humans involved in the operation, and the sampling distance to the animal.

It is useful to distinguish between three types of situations in which collection and use of non-disruptive DNA samples may be desirable. Below we describe: experimental studies, field behavioural studies, and capture- mark- recapture (CMR) research, which may benefit from using non-disruptive DNA sampling.

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Experimental studies

Differentiating, sexing or genotyping individuals prior to experimentation would benefit from using non-disruptive DNA sampling if fitness and behavioural traits are to be subsequently assessed. For example, many species of tropical bird are monomorphic, and can only be sexed using molecular analysis (Vucicevic et al. 2013). Additionally, different species from cryptic species complexes can only be distinguished genetically (e.g. Hebert et al. 2004). Behavioural or fitness studies involving cryptic or monomorphic species may therefore require DNA sexing or identifying of individuals before conducting research on them. Lefort et al. (Lefort et al., 2014; Lefort et al., 2015) used a non-disruptive DNA sampling technique based on the use of larval frass to tell apart the cryptic larvae of two congeneric scarab species. The larvae were thereafter used in feeding experiments, where the effects of the treatment on several of their fitness traits were measured. Even when species identification is not an issue, the organisms being studied may comprise different morphocryptic genotypes (e.g. Fumanal et al. 2005) that need to be determined prior to experimentation.

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 [ADD REF],but such interactions are likely to be disrupted by capture for DNA sampling. [DEVELOP EXAMPLES OF PARENTAL LINKS HERE].

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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 used to estimate population size (Robinson et al. 2009), with the additional benefit of enabling population genetic analysis on the samples collected. Intrusive DNA sampling techniques may affect the survival rate of marked individuals, or introduce avoidance behaviours, which may cause marked individuals to avoid traps, and the population size to be overestimated. For example, toe clipping is commonly used to estimate population abundance of amphibians (e.g. Nelson and Graves 2004), but toe clipping has been shown to affect survival rates in some amphibian species (Mccarthy and Parris, 2004), and stressful collection or capturing method may cause avoidance behaviour from experienced individuals. Such bias can be limited by the use of non-disruptive DNA sampling methods, which have minimal effects on animal fitness and behaviour. 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 (Lampa et al., 2013).

BEHAVIOURAL AND/OR FITNESS IMPACT OF DNA SAMPLING

Reviews of studies examining fitness effects of DNA sampling in non-invasive methods are virtually non-existent. Non-lethal and non-invasive DNA sampling can have unforeseen effects, and the degree of disruption caused to the sampled organism varies greatly with species. When investigated, the fitness consequences of DNA sampling methods have often been measured using survival as an index for fitness (Marschalek et al., 2013; Mccarthy and Parris, 2004; Oi et al., 2013).

Mixed responses have been found depending on the technique and the taxa sampled. Sometimes responses vary strongly between closely related species and even between both sexes of the same species. For instance, wing clipping has been shown to significantly decrease the survivorship in the bumblebee *Bombus melanopygus* (Cartar 1992), while tarsal clipping of the mid or hind leg in *Bombus terrestris* had no

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significant effect on any of the life history traits tested by Holehouse et al. (Holehouse et al., 2003). Another study performed by Vila et al. (2009) showed that neither leg nor hind wing clipping had an effect on the survivorship or reproductive behaviour of adult males of the protected moth *Graellsia isabelae,* while mid leg clipping had a negative impact on female mating success of the same species.

In addition to fitness impacts, some studies have investigated behavioural impacts on species during non-lethal DNA collections. For example, remote biopsy sampling is known to cause little reaction from individuals when conducted correctly, and is unlikely to produce long-term deleterious effects (Bearzi 2000). Nonetheless, all biopsy sampling involves some level of risk (see Bearzi 2000) and different individuals from the same species may react differently to similar stressful situations based on individual physiological and psychological factors including previous experience, reproductive and hormonal conditions, illness, or concurrent pathologies (Barrett-Lennard 1996), age, size (Gauthier and Sears 1999), and gender (Brown et al. 1991). The impact of remote biopsy has been extensively studied on marine mammal behaviour, while not so much in other vertebrates. For example, Gemmell et al. (1997) studied the impact of remote biopsy on male New Zealand fur seal (*Arctocephalus forsteri*) behaviour, and found that in most cases the seal recoiled from the impact and searched briefly for the assailant. Hoberecht (2006) reports that juvenile and adult female Steller sea lions "*Typically turned in the direction from which they were struck and then left the haul-out and entered the water. Many returned to the haul-out within 5 min of being struck by the dart*". Krutzen et al. (2002) found that bottlenose dolphins reacted equally to the darting process regardless of being hit or not, suggesting that the reaction is mainly caused by 'unexpected disturbance' rather than biopsy. No sign of long-term altered behaviours was observed, and sampled individuals were still easily approached for systematic survey and individual tracking.

These examples suggest that no preconceived idea on the invasiveness of a method should be established prior to testing. Therefore it is important to standardise sampling methods prior to defining their invasiveness for a targeted species.

CONCLUSION

Even in cases where organisms are not needed for experiments, there are a number of advantages in having a target organism in hand that is associated with a particular DNA sample as this can be a source of other important data, including morphological, biochemical, physiological, or behavioural observations. We hope that our approach of emphasising the fitness and behavioural effects of DNA sampling methods, rather than whether or not a particular procedure is invasive, intrusive, or injurious in the physical sense, will encourage more research into the effects of different sampling methods on the organism(s) in question. Currently there is a great deal of assumption and supposition in this regard, some of which is tainted with anthropomorphism. We believe that a move to a more empirical evidence-based approach will lead to significant improvements in experimental design, resulting in more robust and repeatable inferences.

TAKE-HOME MESSAGES

1. There is a gap in the current terminology, for which we propose the new term, non-disruptive DNA sampling, to specifically address behaviour and/or fitness effects of a sampling method, rather than physical integrity (invasiveness).

2. DNA sampling methods should be chosen according to the research aims; in particular, non-disruptive DNA sampling should be used 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. Although eDNA and DNA trapping are often regarded as ideal to limit the impacts of DNA sampling on live animals, they are not always applicable, can present technical limitations and are often not compatible with experimental research.

4. If non-invasive methods are very disruptive or stressful to an animal, a more invasive but less disruptive method may be a preferred alternative.

5. More research is required on the fitness and behavioural consequences of different live DNA sampling methods in a variety of species and contexts.

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