

Dear Prof. Sappington,

Please find below our point-by-point answer to the reviewers' comments. Our responses are written in blue. Please note that we provide a track-change version of the manuscript in word format and a final version as pdf. The line numbers given below correspond to the numbering of the track-change version of the manuscript. You will see that we addressed all points raised by the reviewers and have considerably modified the structure of the manuscript. We also added new figures, new analyses and one new section. We hope you will find this version of the manuscript suitable for recommendation by PCI Ecology.

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

**Marie-Caroline Lefort, Robert H Cruickshank, Kris Descovich, Nigel J Adams, Arijana Barun, Arsalan Emami-Khoyi, Johnaton Ridden, Victoria R Smith, Rowan Sprague, Benjamin Waterhouse, Stephane Boyer** <https://www.biorxiv.org/content/early/2018/08/21/385120> version 1

*Submitted by Stéphane Boyer 2018-11-30 13:33*

**Abstract**

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 the 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 58% 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, non-disruptive*

**Round #1**

**Decision** by *Thomas Wilson Sappington, 2019-03-04 21:44*

Manuscript: <https://www.biorxiv.org/content/early/2018/08/21/385120>

**Decision on Lefort et al., "Blood, Sweat and Tears...": Merits revision**

Dear Dr. Lefort,

Thank you for requesting a peer-review of your pre-print, "Blood, sweat and tears...", by PCI Ecology. I apologize for the long delay in providing the reviews, we had some difficulty finding reviewers followed by slow response from one of them.

You will see that the comments of reviewer #1 are favorable. The comments of reviewer #2 are more thorough and critical. They are essentially favorable, but suggest a major revision and restructuring of the paper before being acceptable. Although the requested revisions will require some fundamental changes in how your ideas for a different terminology in the field of DNA sampling from living animals are presented and explained, I think it would be worth the effort, and encourage you to consider doing so. If you can make such revisions, I will be

happy to review the paper afresh and consider for formal recommendation by PCI Ecology.

Please let me know of any questions. I look forward to seeing your revised version. If you choose not to revise, let me know if you would, please.

Best Wishes,

--Tom Sappington

## Reviews

*Reviewed by anonymous reviewer, 2019-02-04 17:03*

In this paper, Lefort et al. review methodology of papers claiming to use non-invasive DNA sampling over the last five years. The authors find that the methodology reported by over half of the papers reviewed do not adhere to the original definition of “non-invasive DNA sampling” proposed by Taberlet & Luikart (1999), which specifies that sampling is non-invasive when DNA is collected without disturbing or catching the animal. These results suggest that the original term has lost its meaning, and rather the term is being used to describe methods that are minimally invasive to the physical structure of the organism but might be extremely disruptive to the behavior of the organism or the organism’s fitness. Two new terms, “non-destructive sampling” and “minimally-destructive sampling” are proposed.

I found the paper very well-written, and the topic important. I believe that the methods used to conduct the review provide a representative glimpse of the recent literature. My major concern is regarding the presentation and definition of the new terms “non-destructive” and “minimally-destructive” sampling. In particular, I found Figure 2 presented contradictory information compared to what is in the text. For example, fecal sampling can be both non-invasive (sensu Taberlet) and non-disruptive if only part of the scat is collected. Or, could be considered non-disruptive when the entire scat is collected but the species is known to be non-territorial and does not use scat for social cues. Likewise, I would suspect that in many cases or for many species, remote-biopsy by darting is disruptive or at least minimally disruptive. Given the apparent gross misuse of the original term “non-invasive DNA sampling”, I think there should be no room for misinterpretation of the newly proposed terms. While Figure 2 needs work, I believe that there are also some inconsistencies in the text (e.g. Ln 402 , below), which together suggest that the new terms might need further refinement and clarification.

We agree with the reviewer here. In fact this could be said of most protocols displayed on this Figure (now Figure 3). The context is essential. As written now in lines 435-436, “Rather than the type of sample, it is the method of sampling that needs to be scrutinized for its invasiveness”. Because of this, it is very difficult to propose any method that would always be non-disruptive or minimally disruptive without providing context. For example the faecal sampling depicted on Figure 3 is meant to show a species that does mark its territory, but it was difficult to also include in the pictogramme the fact that the whole sample was collected. To clarify, we have rephrased some of the text on the figure and now provide a more detailed legend with references for each example, we also stress in the legend that these pictograms are only examples and do not represent an exhaustive list of the existing methods.

Ln 402: I do not agree that non-disruptive DNA sampling is required for laboratory-based experiments. Some experiments require handling or manipulation of the organism that might alter short term behavior and so taking a minimally-disruptive DNA sample at this point might have little added detrimental effect. An example of this would be when animals are collected in the wild and brought to the lab where there is a recovery and acclimation period.

Although we generally agree with the comment of the reviewer, the quote from the manuscript is incomplete here. The sentence reads as follow:

“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”. The crucial point here is that using a disruptive sampling method, **which will affect behaviour and/or fitness** prior to assessing said fitness or behaviour

is likely to introduce bias in the results of the experiment. That said, we agree that an acclimation period could alleviate behavioural impacts in many cases and we have added a sentence about this on line 704-706.

*Reviewed by anonymous reviewer, 2019-03-03 14:01*

The authors are poised to make a valuable contribution to the field of eDNA sampling by suggesting better terminology to replace the use of "non-invasive" for categorizing sampling methodologies meant to not harm (or affect the behavior of) a target animal. The suggestions seem well-conceived and worthwhile. However, the way the paper is structured and conceptually focused around the results of a literature survey causes many unnecessary problems and threatens to derail the authors' main messages. I strongly suggest a thorough restructuring, as outlined at the end of my comments, where the literature survey plays only a supportive role in the authors' suggestions for terminological change. Making the survey the focus of the paper is disruptive to what should be the natural flow of the paper and the authors' arguments.

The following are comments that occurred to me as I read through the paper from beginning to end. I leave them like this, because the problems surfacing in the first half of the paper as currently written highlight the need for structural change recommended in more detail at the end.

What impact does terminology about DNA sampling potentially have on scientific behavior? The authors indicate improper use of "non-invasive" (when it actually could be disruptive) is "misleading" (Line 93). Who is being misled? The reader? The scientists themselves? What are the consequences of using "misapplied" terminology? In other words, is "misapplied" automatically the same as "misleading"? What is the hope of the authors for an outcome of using more precise terminology? Are the authors concerned that use of the term "non-invasive" DNA sampling instead of more accurate "non-disruptive" or "minimally disruptive" will open the eyes of those scientists who use such methods that they may be "invasive" after all and therefore decline to do the experiments (or to change their methodology)? It seems to me the authors need to justify the reason for conducting this study in the Introduction. If it is just to improve labeling of experimental technique for its own sake, then who cares? It would be like taking authors to task for overuse of dangling participles or something. Presumably, the authors have a more compelling reason, but they need to explain it to the reader clearly at the beginning of the paper. They approach this in lines 96-97: "This is problematic for assessing impact on animals, identifying opportunities for refinement, and for ensuring validity and quality of the data collected." But it is not clear how misapplied terminology would have these effects. One would have to assume that the scientists conducting the studies would not take their own methods (including handling of an animal for instance), whatever they may call them, into account when interpreting their data, and that this would be caused by their use of incorrect terminology. Is this what the authors mean? If so, they'll need to provide some examples, because it sounds implausible. If not, they will need to explain more precisely what they do mean.

We tend to agree with the reviewer that it sounds implausible (or extraordinary rare) that authors ignore potentially important effects of their sampling method on their results. However, further application of the sampling methodology by other end users, may be problematic. When non-invasive DNA sampling is misapplied, the reader (especially the naive reader) may be misled. This includes decision makers, conservation managers, and other end-users who may be unfamiliar with the scientific literature on DNA sampling. Using more precise terminology (such as "non-disruptive" or "minimally disruptive") could also, as suggested by the reviewer, open the eyes of those scientists who use so-called "non-invasive methods" that they may be "invasive" after all, and encourage them to consider reducing the impact of their sampling or search for alternatives. As pointed on lines 249-251, there are many truly non-invasive methods that have been published, but are very rarely used.

The above justification is now stated in the introduction on lines 230-233.

L44: change "on the behaviour" to "on behaviour"

Done

L71: change to "...prior to experimentation with the same individuals,..."

Done

L80-84: So are the authors saying that the only non-invasive DNA is eDNA? That they are synonyms?

First, there is no such thing as "non-invasive DNA". There is DNA that is collected non-invasively. This is a crucial point, and a very common shortcut used by many authors. We tried to make this point under the section "Misclassification of faeces as non-invasive DNA samples", now on lines 434-435: "Rather than the type of sample, it is the method of sampling that needs to be scrutinized for its invasiveness". We are extremely concerned that this point has not come across and have reworded the text to try and clarify further.

It follows that "non-invasive DNA sampling" and "eDNA sampling" are not synonymous because there are many examples of eDNA that can be collected in an invasive way (again sensu Taberlet et al.), for example by collecting the faeces of an animal that use excrements to mark its territory. In addition, non-invasive DNA sampling (sensu Taberlet) may also include DNA trapping when this is conducted with unbaited traps (see Figure 3).

L103: Replace "(" with a comma after "noninvasive"

Done

L107-108: the search term "ecology" is listed twice in the same string

Corrected

Method section: The authors confine their description of methodology to how they conducted their literature search. However, they must also include methodology for deciding how they classified use of terminology in the papers examined. In other words, how exactly did the authors decide if a particular way of collecting DNA in a paper was non-invasive or invasive? How were categories assigned (i.e., what rules were followed), related to categories like "potentially affecting territory" or "definitely invasive" or "contact", as in Fig. 1? These rules may seem obvious to the authors, but they must be explicitly stated or described so there is no room for misunderstanding by a reader.

We did provide description of the different categories in the legend of Figure 1, now Figure 2. However, we contend that this was not sufficient. We have improved this legend for greater clarity and have also included a description of the general rules used in the method section. In short, we extracted information from the method section of each paper revealed by the systematic search, compared them against the definition of Taberlet et al. and decided whether or not they complied with the definition. We only used papers for which information was provided (342 papers in the final dataset). We added an intermediary category labelled as "potentially affecting territory", for cases where faecal samples were taken from wild animals that are known to use dejections as territory or social marking. In addition, we now provide our raw data in a table containing the list of papers and how each of them were categorised (Supplementary Table 1).

Section 3: "Seven deadly sins" and subheadings listing "Sin 1:", "Sin 2:", etc. ; the use of the word "sin" like this in a scientific paper seems inappropriate and will be offensive to some, probably many readers. I assume the authors mean it in a catchy, jaunty, word-play way, but I strongly recommend removing that language. It is hyperbolic with pejorative connotations, and gives the impression of prejudging the motivations of authors who have used the term "non-invasive" incorrectly up to now. Avoiding this kind of judgmental language will also improve the impact and future acceptance of the paper's ultimate recommendations for alternative, more-precise terminology by these same and other authors.

The "seven deadly sins" or the "seven sins" is a very common phrase that has been widely used in the scientific literature. A search on the ISI WebOfKnowledge for these terms in the titles only yields 295 papers across all scientific fields, totalling 1973 citations. It is clear that this phrase is common language. Rather than an attempt to offend readers, the use of this phrase highlights the fact that many authors use the term non-invasive DNA sampling improperly while it has been very clearly defined and its definition is (or should be) well known. We would like to emphasise that our intention is not to be judgmental, or dismiss other studies. For example, we specifically acknowledge the fact that in most cases, authors tried to minimise the impact of sampling on line

518. We merely observe that there is a significant issue with the use of this terminology and we believe that as a scientific community, we need to do better. To avoid over-dramatization, we dropped the term “deadly” and now only use “seven sins”.

L150-153: The example of incorrect terminology is for earthworms, but the authors then state, "Yet, truly non-invasive methods exist for invertebrates" citing papers involving only arthropods (except, even more oddly, ref 19 seems to be about fish based on the title). It would seem more appropriate to use a different example of incorrect terminology based on an insect or some other arthropod rather than an annelid. Insect exuviae, pupal cases, etc. are not relevant as possible non-invasive sampling methods for earthworms, but the sentence is written as if they are.

This paragraph (now lines 249–251) relates to non-invasive DNA sampling in invertebrates. Therefore the citation about fish is indeed erroneous and has been replaced by one on aquatic macro-invertebrates. The earthworm study cited in the third sentence of this paragraph is a particularly striking example of an invasive method applied to invertebrate and labelled as non-invasive. All examples listed in the fourth sentence relate to DNA sampling in invertebrates in general and did not include a method for earthworms. There are of course truly invasive methods for earthworms as well, and we have now added one example.

L160-162: "It is difficult to imagine...": delete this sentence. It is subjective speculation and judgmental of motives, and is inappropriate in objective scientific writing.

We removed this sentence.

L171: change "animal ware" to either "animal was" or "animals were"

Done

L171-174: The authors' description of the Kierepka et al. (25) study is problematic and disturbing, because it misrepresents what was done in that study. They state, "For example, Kierepka et al. [25] obtained faecal samples from feral pigs (*Sus scrofa*) by culling the animals and squeezing faecal pellets out of the pigs' rectum shortly after death. Such procedures clearly violate the definition proposed by Taberlet et al. [6]..." It sounded strange why anyone would be so foolish as to claim a sample was non-invasive when taken from an animal killed expressly for taking that sample, so I looked up the paper. The animal studied is an invasive pig that is controlled in DOE's Savannah River Site (SRS) to protect the habitat and native wildlife. The purpose of the study was NOT to sample the pigs non-invasively, so it is not even relevant to Taberlet's et al. definition. Instead, it was a methods development paper involving 1) development and testing of microsatellite markers that would work for this species after sampling DNA from feces; the experimental design was to compare marker amplification from actual tissue (positive control) versus from feces from the same individual of known sex, age, etc.; and 2) how well such sampling would work at time intervals after exposure of feces to the warm moist climate of the southeastern U.S. under which DNA degradation could be rapid. Kierepka et al. were developing methods for FUTURE non-invasive sampling. **They never claimed that they were taking non-invasive samples in this study itself.** So to characterize it that way is clearly misrepresentative, and the paper should not even be included in this study, much less used as an example of bad behavior. I cannot take the time to examine every paper the authors cite as violating "the definition of Taberlet et al." But I am concerned about how many other fundamental errors in classifying the papers there may be. Kierepka et al. is simply a paper that is irrelevant to the study undertaken in this preprint. How could the authors make such an obvious error? It is also unclear what the authors are trying to accomplish with their criticisms of misapplication of "non-invasive" terminology, as described above. I'm getting the impression it is driven at least partly by a vague sense of being critical of "hurting" other animals and misusing terminology to cover it up, even though in this case these are invasive pigs that are apparently routinely culled to protect the SRS, and even though Kierepka et al. affirm that, "All trapping and euthanasia methods conformed to the American Society of Mammalogists guidelines (Sikes et al. 2011) and University of Georgia Animal Care and Use Committee policies (Protocol 12-010-Y3-A4)." Not hurting other animals is a laudable sentiment and worthy of study. But it is all mixed up in this study to the point where the authors' immediate goal regarding their purpose is unclear, perhaps even to themselves, resulting in methodological confusion.

We agree that the study from Kierepka et al. was not accurately described. This study was selected in our literature search because the words “noninvasive”, “DNA” and “sampling” are present in the abstract or the keywords. However, our screening of the method section for this paper was incorrect in that the authors do not claim to use a noninvasive DNA sampling method, they merely refer to other studies that do so. In addition, our wording was particularly poor because it suggested that the animals were culled with the aim of taking DNA samples. This was a gross misinterpretation as the animals were actually culled for another reason and DNA samples were collected opportunistically upon culling. We are grateful that the reviewer has checked this particular reference and we removed it from our final dataset. Following this mistake, the method section of all articles were screened again and we now cite the correct reference (Kolodziej et al. 2013) in the corresponding section. The wording was also modified to ensure it accurately describes the study by Kolodziej et al. A second example was also added to the section, which is now on lines 266-435.

We would like to reiterate that our aim is not to stigmatise or being judgmental towards authors, but to inventory the methods used and bring to light the existence of widespread issues in the current literature.

L207-215: How did the authors of the current paper classify these studies? Where do they fall in Fig. 1? The classification methods should be described in the Method section.

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. When one of the method used was invasive but this was not specified by the authors, the papers were classified as non compliant with the definition by Taberlet et al. The rationale is that naive readers will be unable to differentiate between invasive and non-invasive sampling methods unless they are provided with clear information for each of the different methods used. Again, this represents only 31 papers out of 342

Details of the classification for each paper are now available as supplementary data. More information has also been added to the legend for Figure 1 and to the method section.

L216-219: The authors actually describe a situation like that above for the Kierepka et al. paper, where invasive sampling is used for methods development of non-invasive sampling. It is not clear why this falls under the scope of the current pre-print. Do those papers claim that the invasive sampling is non-invasive? If not, the papers do not seem relevant to the question of correct terminology.

These studies fall in the scope of the review because they contribute to the confusion around the definition of non-invasive DNA sampling. In particular, when authors don't specifically state that the method as used in their paper is invasive, or do not provide a protocol for non-invasive sampling that could be combined with the novel molecular analysis they propose. This contributes to the common misconception that the type of sample collected is either invasive or non-invasive when in fact, it is the method of sampling that needs to be scrutinized for its invasiveness.

Section 3.6: Why is this a "sin"? And who is "guilty" of it? Taberlet et al. for creating an "all or nothing" definition of non-invasive sampling? Again, the purposes of the study are muddled.

The “all or nothing” definition proposed by Taberlet may not be ideal, but it is the authors that misuse the term non-invasive DNA sampling. When there are no middle ground, authors should define one. This is what some authors (six papers in our database) who decided to use the term “minimally invasive”. The purpose here is to encourage all authors to use the latter term, “for which potential impacts of the sampling are acknowledged, while still emphasising the aspiration of the authors to minimise those impacts”

L280-281: use of the veterinary sense of non-invasive "...could minimise the perceived impacts of sampling methods on animal welfare, even if these impacts are significant in reality." Perceived by whom? The authors are either assuming the researchers in these studies using this alternative definition will not take into account animal handling when they interpret their own data, or they are worried other readers will not recognize the same, or that the researchers are covering up the possible impacts on animal welfare by using this different definition. Still not clear what the authors' concerns about impacts of use of terminology are.

We now list our main concerns on lines 230-238:

“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 (such as "non-disruptive" or "minimally disruptive") 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 last half of the paper is much more straightforward and potentially useful to the community of researchers than the first half. My recommendation is to restructure the paper to start with a review of current methods used to take DNA samples from wildlife with the purpose, to some degree or other, of leaving the animal to carry on with its life in the wild. Then introduce the problems associated with the use of "non-invasive" terminology introduced by Taberlet et al. (including veterinary-style use of the term) as revealed by the literature analysis. Then end with the suggestions for new terminology.

Our original plan was not very far from the proposed structure. For memory, the original structure was:

1. DNA collection and the non-invasive misnomer [Introduction to the problems associated with the use of non-invasive terminology introduced by Taberlet et al.]
2. Methods [methodology used for the literature review]
3. The 7 sins of non-invasive DNA sampling [Details of the problems associated with the use of non-invasive terminology introduced by Taberlet et al.]
4. Introducing the terms non-disruptive and minimally disruptive DNA sampling [suggestions for new terminology]
5. When is non-disruptive required or preferred
6. Take home message

We modified the introduction to make it more general and less focused on the current methods used and less on the terminology issue. We also added a general section on the methods used for non-invasive DNA sampling from wildlife

as suggested by the reviewer. Our new plan is:

1. Introduction [general introduction on methods used for non-invasive DNA sampling from wildlife]
2. Methods [methodology used for the literature review]
3. What non-invasive DNA sampling is used for [Review of the methods used for non-invasive DNA sampling from wildlife]
4. DNA collection and the non-invasive misnomer [Problems associated with the use of the non-invasive DNA sampling terminology introduced by Taberlet et al. (with reference to Box 1)]
5. Introducing the terms non-disruptive and minimally disruptive DNA sampling
6. When is non-disruptive required or preferred
7. Take home message

In other words, the paper is most naturally a review of how DNA samples can be taken when a researcher's goal is to interfere as little as possible with the target animal's current and future well-being and activity in nature, and the pitfalls of doing this incorrectly or of making naive assumptions about impact on the animal. However, the paper currently is structured as if it is chiefly a research paper, with the analysis of terminological use of "non-invasive" in the literature as its centerpiece and most valuable aspect; and that "discovery" of misapplied terminology through this survey analysis demands a change. Focusing the paper on the literature search analysis is a mistake that makes the logic hard to follow and muddies the authors main messages which do not actually rely on the results of the analysis. These are: reminding researchers to be careful in assuming that just because they don't injure an animal when sampling doesn't mean taking the sample has no negative impact on the animal or its behavior; that even samples taken without contacting the animal (such as of fecal material) if



done heedlessly could interfere with future animal behavior if that material plays an ecological or behavioral role, for example, in marking territory; and that better terminology based on more clear thinking about the effects of DNA sampling methods on the animal will help the reader more clearly and realistically evaluate a researcher's interpretation of their data. Analysis of the literature search can and should serve to buttress the authors' appeal to the scientific community to adopt better and more appropriately attributed terminology than has been used heretofore. That analysis is best presented as illustrative of the authors' main points, not the center of attention around which the rest of the paper revolves.

The appeal for better terminology as suggested by the authors seems valuable and reasonable. If the authors can restructure the paper, as well as address the many issues in the first half of the paper as described above, the authors' suggestions could have a major impact on the field. In my best judgment, leaving the paper's structure as is will probably result in little or no impact.

The reviewer makes a number of very good points with which we generally agree. However, we also think that for many readers, the literature review is not simply supportive to our main argument, but absolutely essential. To give some context, we wrote the first version of this manuscript in 2015 and originally we did not have a systematic literature review. When submitting our manuscript to several journals, virtually every review we received pointed that the term non-invasive DNA sampling had been defined years ago, that everybody knew what it meant and that misuse in the literature was marginal at most. We strongly believe that the results of our literature review will come as a surprise to many, and that scientists need to realise how widespread the issue is before they can support the proposal for more precise terminology. We propose to summarise the main issues exposed by our literature review in a standalone box. This way, readers will be able to go over the paper without spending time on the details of the literature search if they choose to.