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Review

Inside the Redbox: Applications of haematology in wildlife monitoring and ecosystem health assessment

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HIGHLIGHTS

- Non-lethal sampling procedures are necessary in wildlife monitoring
- Haematology is still an opaque science for field studies
- Recent advances in technology widen the possibilities for blood samples in bio-monitoring
- Users should follow some rules to increase the diagnostic value of blood samples
- Data should be submitted to a public repository as a requisite for publication

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ABSTRACT

Blood analyses have great potential in studies of ecology, ecotoxicology and veterinary science in wild vertebrates based on advances in human and domestic animal medicine. The major caveat for field researchers, however, is that the 'rules' for human or domestic animal haematology do not always apply to wildlife. The present overview shows the strengths and limitations of blood analyses in wild vertebrates, and proposes a standardisation of pre-analytical procedures plus some suggestions for a more systematic examination of blood smears to increase the diagnostic value of blood data. By discussing the common problems that field researchers face with blood variables, we also aim to highlight common ground enabling new researchers in the field to accurately collect blood samples and interpret and place their haematological findings into the overall picture of an ecological or eco-toxicological study. Besides showing the practicality and ecological relevance of simple blood variables, this study illustrates the suitability of blood samples for the application of cutting-edge analytical procedures for expanding the current repertoire of diagnostic tools in wildlife monitoring and ecosystem health assessment.

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1. Introduction

Studies on the response of vertebrates to natural and anthropogenic stressors are vital for the development of effective strategies to stem biodiversity loss and to monitor ecosystem health (Jorgensen, 2011; Todgham and Stillman, 2013; Maceda-Veiga et al., 2014). As apical consumers, vertebrates reflect human-induced changes in below trophic levels including the presence of pollutants (Pérez et al., 2008; Ramos and González-Solís, 2012; Monroy et al., 2014). Given the similarities in their metabolic pathways, the risk posed by pollution, including emerging contaminants (see Pal et al., 2010), in vertebrates can often be predicted by examining the health consequences for single taxa (Huerta et al., 2012), although the physiological response may also vary depending on individual traits (e.g. sex, species) (Evans, 2008; Cabarcas-Montalvo et al., 2012). Health diagnostics in wildlife can also increase our understanding of infectious disease dynamics, including those with human interest, such as *Campylobacter*, rabies virus and West Nile (Colles et al., 2011; Serra-Cobo et al., 2013; Arnal et al., 2014). Thus, studies of conservation, toxicology and epidemiological sciences benefit for generating in-depth knowledge of the physiological response of wild vertebrates to biotic and abiotic factors (Wikelski and Cooke, 2006; Benskin et al., 2009; Cooke et al., 2012). Currently, however, such physiological studies need to be sensitive to new animal welfare standards and the conservation status of many vertebrate taxa (Sikes and Gannon, 2011; Maceda-Veiga, 2013; Thrusfield, 2013).

Recent advances in technology widen the possibilities of non-lethal sampling for gathering physiological data in vertebrates through the use of electronic devices attached to animals (biologging, Cooke et al., 2004) and the examination of non-lethally collected samples, such as faeces, hair, urine, feathers and blood (Bortolotti et al., 2008; Berkvens, 2012; Narayan, 2013). Peripheral blood is probably the most informative tissue that can be non-lethally sampled in vertebrates as it can reflect whole-organism function (Douglas et al., 2010). In an attempt to familiarise general practitioners with the use of haematology in wildlife studies, this overview shows the practicality and ecological relevance of blood variables along with the advantages of incorporating standardised procedures and cutting-edge technology into blood analyses in wild vertebrates.

2. The Redbox tool kit

Blood is an aqueous fluid (plasma) containing red blood cells (RBCs), white blood cells (WBCs), and platelets (or thrombocytes) that circulate through the vascular system carrying oxygen, nutrients and a vast range of metabolites/electrolytes to and away from tissues (Douglas et al., 2010). Consequently, the list of potentially useful blood variables for wildlife monitoring is almost as long as the number of blood components (hereafter 'blood markers'; Tables 1, 2 and 3). However, careful selection of blood markers is needed since they differ in the information they provide, analysis costs, and sensitivity to the time-lag from blood collection in the field to their determination in the laboratory. This selection is particularly important when the objective is assessing the health status of an individual, especially in small sized species given the reduced volume of blood available. In this regard, a blood smear only requires 5–10 μl of blood, a comet assay can be done with less than 2 μl of blood, and a full biochemistry profile can be obtained with 100 μl of blood using the VetScan® Avian Reptilian Profile Plus (Abaxis,

Table 1
Diagnostic value of common changes in the colour and shape of red blood cells (RBCs) observed in blood smears. The taxa column indicates to which vertebrate group the diagnostic value can be applied (M = mammals; B = birds; R = reptiles, and F = fish) following authors' experience, and Douglas et al. (2010), Pendl (2013) and Wolfensohn and Lloyd (2013).

Observation	Description	Pathological indication	Taxa
RBC morphology and colour			
Hypochromy	The colour of RBC is pale (i.e. low haemoglobin concentration)	Anaemia, iron deficiency	M, B, R
Poiquilocytosis	Modifications in RBC morphology	Metabolic dysfunction (e.g. an extended RBC crenation indicates plasma hyperosmolarity in mammals) and increase in erythropoiesis rate	M, B, R
Anisocytosis	Modifications in RBC size	Metabolic dysfunction and increase in erythropoiesis rate	M, B, R
Anisocariosis	Different nuclear shapes and forms amongst the cells	Malnutrition and septic disorder	R
Sphaerocytes	Case of poiquilocytosis in which RBCs are circle-formed	Haemolytic anaemia	M
Hedgehog erythrocytes	Case of poiquilocytosis in which RBCs have pointed cytoplasm	Uremia, hyperthyroidism and hypertonicity	M
Howell–Jolly corpuscles		Abnormalities in nuclear division, hyposplecnic	M, B, R
Basophilic stippling		Iron deficiency and Pb intoxication	M, B, R, F
Heinz corpuscles		Exposure to haemoglobin oxidation and enzymatic dysfunction	M, B, R, F
'Rouleau'	RBCs form "piles of coins"	Inflammation	M
Haemoglobin non-homogeneously distributed	The colour of RBC is not uniform		M
Cabot ring	Denaturalization of a protein for the cytoplasm layer	Unknown	M
Intra-cytoplasmic corpuscles	Cytoplasmic corpuscles with internal nucleus	Lizard erythrocytic virus and haemoparasites	R, B, M
Vacuolization of cytoplasm		Unknown but also artefactual	M, B, R, F
Intranuclear crystals	Crystallization of haemoglobin	Anticoagulant failure (especially EDTA or citrate)	R
RBC agglutination	RBC stick to each other	Anticoagulant failure leading to false HCT and total RBC count	

Table 2
Diagnostic value of the results from the differential white blood cell counts in blood smears across vertebrates following authors' experience, and Davis (2009), Douglas et al. (2010), Martínez-Silvestre et al. (2013), Pendl (2013) and Wolfensohn and Lloyd (2013). Note that thrombocytes and the special granulocytic cells (see SGC in the main text) are not listed due to the fact that their immune function is under debate.

WBC	Observation	Diagnostic
Neutrophils or heterophils	↑	Stress, infection, tissue damage, inflammation and metabolic dysfunctions but normal in some camels and possibly in raptors
	↑Immature cells or with toxic granulation[a]	Inflammation and infection
	Ring shaped nuclei	Inflammation in carnivorous but normal in some rodents
	Mitosis	Acute infection
Eosinophils[b]	↑	Parasitic infection but normal in camels and ambystomatid salamanders
	↓	Stress
Basophils[c]	↑	Tissue damage, inflammation and parasitic and non-parasitic infections but normal in elephants
Azurophils[d]	↑	Tissue damage, infection and inflammation but high numbers in healthy snakes
Lymphocytes	Mitosis	Acute infection, neoplasm (mammals and birds) and whole body bacterial infection (reptiles)
	Phagocytosis	Whole body bacterial infection
Monocytes	Mitosis	Acute infection (reptiles)
	↑	Tissue damage, chronic inflammation and infection

[a] Dark and coarse granules are present particularly in neutrophils along with cytoplasm vacuolization.
[b] Rare in felines and horses, and may not be present in some fish species.
[c] May not occur in some fish species.
[d] Only in reptiles (mainly in snakes and lizards).

Inc.). In health diagnostics, however, the major caveat with blood analyses is the lack of baseline data for many wild vertebrates. At best when exist, practitioners determine the health status of individuals by comparing their blood profiles to those reported in literature. Nonetheless, such comparison is mostly difficult due to the discrepancies in the way haematological data are gathered and/or reported in publications (see Davis et al., 2008).

As the variation due to pre-analytical procedures (e.g. collection and storage) in haematological studies is usually much higher than the variability associated to analytical techniques (Evans, 2008), we propose a standardised pre-analytical procedure for blood analyses in wildlife plus some suggestions for a more systematic examination of blood smears (see details in Fig. 1 and Appendices A and B). Like other disciplines (e.g. genetic studies and GenBank), we also suggest that the submission of the blood data to a public physiological data repository (e.g. International Species Information System, the National Ecological Observatory Network in USA, the National Wildlife Health Database project in New Zealand) should be a requisite for publication as this would facilitate cross-study comparisons and meta-analyses. In this regard, a public depository developed by the University of Georgia compiles differential WBC count across taxa, but fish are still not included (see Davis, 2009). With current advances in image analysis software, cross-study comparisons could also be facilitated if public depositories enable including full microscopic views of blood smears (see telepathology in Goswami et al., in press). As these proposals are likely to be a long-term achievement, we propose concentrating efforts now in standardising how data is reported in publications (see Appendix C) and making micrographs of novel materials electronically available at least in each publication as Supplementary material. Besides the use in veterinary medicine, public physiological depositories can be useful to collect blood data from studies of ecology and toxicology. The sections below show ethical issues and a compendium of applications of some of the most common and informative blood markers used in these disciplines along with their sensitivity to fieldwork constraints.

2.1. Animal welfare and challenges of blood sampling in wild vertebrates

Blood sampling from live animals is considered an experimental procedure in the European Union, Canada and USA so animal welfare

Table 3
Diagnostic value of common biochemical variables determined in the plasma of vertebrates following authors' experience, and Douglas et al. (2010), Martínez-Silvestre et al. (2013), Pendl (2013) and Wolfensohn and Lloyd (2013).

Plasma biochemistry	Stress	Liver damage	Gota' visceral	Kidney disease	Muscular damage	Necrosis	Septicaemia	Inflammation	Poor nutrition	Dehydration	Pregnancy
Cortisol	↑↑↑										
Total proteins		↓				↑[b]		↑↑–↑[b]			↓
Glucose	↑↑↑						↓		↓		
Uric acid[a]			↑↑↑[b]	↑[c]							
Urea[a]		↓ (severe)		↑–↑↑↑							
Creatine											
Bilaverdin											
Bile acids		↑		↓[b]							
Triglycerides		↑↑↑							↓		↑
Cholesterol		↑		↑							↑
Aspartate-transaminase		↑↑↑–↑[b]		↑[b]	↑↑↑	↑	↑↑↑	↑			↑
Alanine-transaminase		↑↑↑–↑[b]		↑[b]	↑						
Lactate dehydrogenase		↑↑↑–↑[b]		↑↑↑[b]	↑↑↑		↑	↑			
Alkaline phosphatase		↑↑↑–↑[b]									↑[b]
Creatine kinase	↑↑↑	↑[b]			↑↑↑	↑↑↑					↑
Sodium									↑	↑↑	↑
Chlorides				↑					↓	↑	
Potassium								↓	↓	↑	
Calcium				↑[b]							
Phosphorous				↑↑↑		↑[b]					

[a] Except for amphibians and fish.
[b] Reptiles.
[c] Birds.

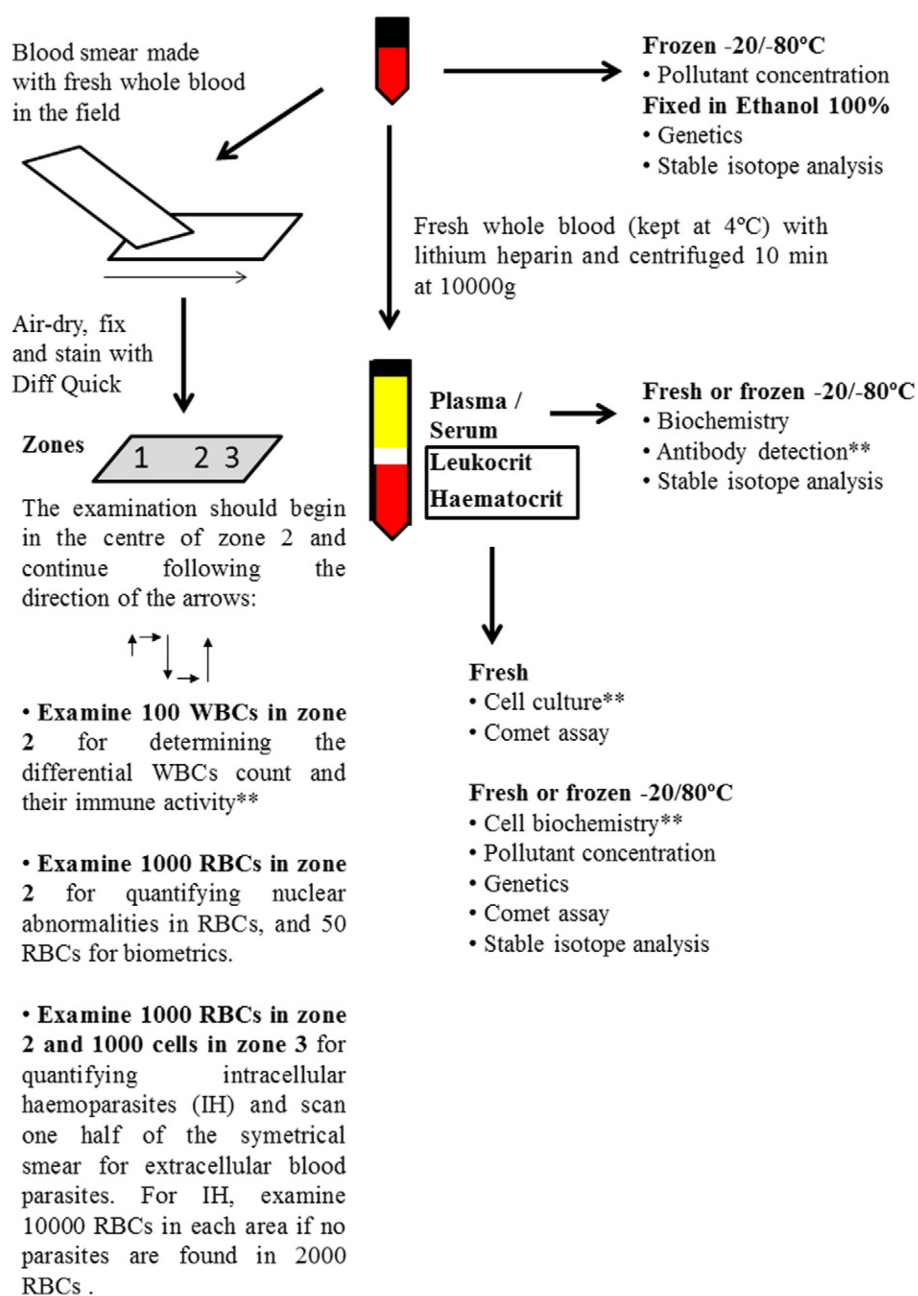

Fig. 1. Work flow of pre-analytical procedures and blood smear examination for the determination of common blood variables in vertebrates following author's experience, Davis and Hopkins (2013), Douglas et al. (2010), Ehret et al. (2002), Hobson et al. (1997) and Pendl (2013). The use of anticoagulants is not recommended for the blood analyses marked (**). Studies with any modification in this pre-analytical procedure should come along with a methodological comparison to facilitate cross-study comparisons. See Appendices A and B for further details.

committees must approve its use in research, with the exception of blood samples obtained from standard veterinary practices and from hunted animals (Evans, 2008; Wolfensohn and Lloyd, 2013). Whilst target taxa have several venipuncture sites (see Appendix A for further details), practitioners must be consistent with their choice (at least in each study) as readings for some blood biomarkers can differ between venipuncture sites up to 70% (see López-Olvera et al., 2003). The general rule for a single blood extraction in vertebrates is not to take more than 5–10% blood volume (maximum volume) of a given animal, for example a 1 kg animal could yield 10 ml of blood (Evans, 2008; Wolfensohn and Lloyd, 2013). In multiple bleeding, this percentage decreases depending on the bleeding frequency and blood component turnover, which vary between species but with some general rules. Plasma turn-over is shorter than that of RBCs for which the average life span is shorter in mammals (~120 days) than in vertebrates with nucleated RBCs (up to more than 600 days) (Douglas et al., 2010; Wolfensohn and Lloyd, 2013). If the RBC life span is unknown for a given mammal, it can be estimated by using the formula suggested by Vácha (1983): mean RBC life span (in days) $= 68.9$ (body weight)$^{0.132}$.

In multiple bleeding, the general rule across vertebrates is to not sample more than 1% of an animal's circulating blood every 24 h (Wolfensohn and Lloyd, 2013). Besides an ethic conflict, the imbalance between bleeding frequency and RBC reposition causes an artefactual decrease in RBC counts (phlebotomy anaemia). It should be noted that our indications for the safety blood collection in wild vertebrates follow animal welfare standards established in laboratory species. The response to bleeding may, however, differ in wild animals, which are also exposed to more stressors than captive individuals such as predation, long-migration and dehydration. Thus, we encourage field researchers to conduct pilot studies to test whether their blood sampling procedures

affect the performance of target species, and always collect the minimum amount of blood possible. To minimise any potential effect on animal health, our suggestion is to never collect more than 80% of the maximum blood volume allowed following standards for laboratory animals, and even reduce it by 25% in very active animals (e.g. birds) or if other experimental procedures are carried out (e.g. collection of regurgitates).

The second challenge of blood sampling in wildlife is the influence of handling stress. Any trapping method (e.g. nets, cages, electrofishing) and/or animal immobilisation for bleeding generate a stress response with short or long-lasting effects on many haematological variables (Peinado et al., 1993; Pagés et al., 1995; Breuner et al., 2013; Krause et al., 2014). As stress hormone levels are affected within minutes of handling, their pre-capture levels are extremely difficult to determine in wildlife (Romero and Reed, 2005). The hormone-induced peak in the remaining blood variables (e.g. glucose concentration, WBC and RBC counts), however, lasts hours in most vertebrates (Davis et al., 2008), although a shorter time-lag (30 min) has been reported in some bird species (Cirule et al., 2012). Interestingly, animal's stress can be measured indirectly by other markers that are not affected immediately by this peak in stress hormones, such as WBCs (see later). The influence of stress hormones on other blood variables can be minimised by capturing animals using sedatives (e.g. large mammals) (reviewed by Kreeger and Armeno, 2007). A careful selection of sedatives is, however, needed since these drugs may alter plasma chemistry, and some taxa, such as felines, are particularly sensitive to them (Sikes and Gannon, 2011). In some experimental designs, animal capture for bleeding is not required since automated devices collect blood whilst animals are resting (Becker et al., 2006). Haematophagous parasites present in animal's nests also provide blood samples suitable for genetic and even, in certain parasite species, for biochemical analyses (Alcaide et al., 2009; Markvardsen et al., 2012).

The final challenge for blood collection in the field is sample preservation until analysis. Given the perishable nature of blood, adding anticoagulant and keeping the blood samples cold (4 °C, without direct contact to ice) are mandatory for any measurement related to blood cell numbers and/or plasma (Evans, 2008). Based on the information collated from published studies across taxa, lithium heparin seems to be the anticoagulant with lowest risk for causing artefacts in blood samples. For example, sodium heparin alters plasmatic ionic composition and EDTA changes RBC morphology and interferes with the anaesthetic (MS-222) commonly used in fish (Walencik and Witeska, 2007; Douglas et al., 2010). In addition, EDTA based anticoagulants can give misleading high plasma levels of metals such as lead due to its selective extraction from RBCs (see Smith et al., 1998). Even when anticoagulant is used, blood must be processed quickly after blood collection to ensure data quality. For plasmatic variables, blood samples need to be centrifuged and, if not analysed immediately, frozen at −20 or −80 °C (but see Arizmendi-Mejía et al., 2013). Artefactual morphological changes can also occur in blood cells even if blood samples are stored at 4 °C (see Vives-Corrons et al., 2014). Nonetheless, blood smears can be easily done and fixed in methanol after air-drying in the field (but see Fig. 1). From our experience, flies or high humidity can degrade blood films in the field, so slides may need to be kept in cabinets and then the drying process can be helped by using dehumidifiers, indirect heating (20 °C) or fans provided with air-filters to prevent dust deposition. Further, to minimise artefactual changes in blood cell characteristics, we recommend staining and doing the slide mounting upon arrival to the laboratory. The staining properties of WBCs are likely to change in old-fixed smears making cell identification difficult.

Together with traditional haematological analyses, blood samples are suitable for other techniques, such as stable isotope and genetic analyses including epigenetics, for which blood only needs to be immediately frozen or fixed in ethanol in the field (Fig. 1). If high-quality DNA is needed or the target is RNA, samples should be kept frozen at low temperature (see Akor-Dewu et al., 2014; Head et al., 2014) or preserved in RNAlater (e.g. Weber et al., 2010), FTA® cards (e.g. Mendoza et al., 2012) or PAX gene blood tubes until analysis (e.g. Thach et al., 2003). In this regard, we also recommend preserving several aliquots of blood per individual instead of a single large volume. For stable isotope analysis, chemical preservatives such as ethanol can bias stable isotope signatures (Bugoni et al., 2008). These authors proposed air-drying as a practical and unbiased method for blood preservation in field studies where freezing is not a practical option. Air-drying, however, may not be feasible in high moist conditions, especially with large blood volumes. If chemical preservatives are used, we recommend comparing chemically and non-chemically preserved blood samples from the same or similar species, and reporting the mathematical correction so as facilitates cross-study comparisons.

2.2. Blood analyses: opportunities and complexity

The cheapest but highly informative option to obtain information on individuals' health from blood is through the examination of blood smears (also known as 'blood films', Fig. 1 and Appendix B). Differential WBC count (DLC, also named 'leukocyte profile' or 'leukogram') is probably one of the most popular markers that can be obtained from blood films (Table 1), being its simplified version, i.e. the relative proportion of neutrophils (or heterophils) to lymphocytes (hereafter, N:L ratio), widely applied in ecological studies of vertebrates as an approximate measure of stress or innate immune response (reviewed by Davis et al., 2008). A major drawback with this method is that it gives a too coarse picture of the immune response ignoring other WBC types, such as monocytes, that play a key role in immunity or even are the predominant WBC type in certain taxa, such as in some fish species (Davis et al., 2008). The wide application of the N:L ratio also makes cross-study comparisons of WBC profiles amongst taxa difficult, but most importantly, hinder the possibility to re-calculate published DLC due to changes in the nomenclature of granulocytes over time due to advances in knowledge about their function especially in fish, amphibians and reptiles (Ainsworth, 1992; Douglas et al., 2010).

Nomenclature is assigned to vertebrate granulocytes on a functional and morphological basis with a trend amongst researchers in using mammalian granulocytes as a model (e.g. Ainsworth, 1992). Different names are used for granulocytes that did not resemble those of mammals such as heterophils in birds and reptiles (Hawkey and Dennet, 1989), azurophils in reptiles (Hawkey and Dennet, 1989) and special granulocytic cells (SGCs) in some fish species (Tavares-Dias et al., 1999; Maceda-Veiga et al., 2013). To calculate the N:L ratio, the problem arises when these cells are considered as a neutrophil-like cell as occurred with azurophils in the past (Hawkey and Dennet, 1989), and currently could happen with SGC (also named PAS-positive granulocytic cell). Despite their function is still unknown (Tavares-Dias, pers. com.), changes in SGC numbers occur as a general stress response like neutrophils but also happen during parasitic infections (Ranzani-Paiva et al., 2000; Garcia et al., 2007) as eosinophils often do. Morphologically, SGCs have a cytoplasm with abundant granules that resemble heterophils but their granules are reportedly to differ in cytochemical properties (Tavares-Dias, 2006). Such nomenclature controversies are, however, unlikely to have a major repercussion for the diagnostic value of differential WBC count as long as all WBC types found in each species are enumerated and the percentage per cell type reported in publications. Besides increasing baseline data available on WBC profiles (see Appendix C), this will improve our knowledge of how each WBC type responds to diseases and/or environmental stressors.

Together with DLC, blood films enable us to determine RBC profiles, which are useful for determining the presence of intraerythrocytic parasites, the toxic effects of pollutants and the oxygen carrying capacity of vertebrates (Fig. 1; Table 1). For quantifying cellular damage, the proportion of RBCs with nuclear anomalies can be determined along with the percentage of dead RBCs (see ecotoxicology section). Another measurement in RBC profiles is the percentage of immature circulating RBCs indicating acute stress response and blood cell production rate.

Impairments in RBC formation can also be detected by comparing RBC size (see Appendix D), which can be used as a proxy measure of organism's aerobic ability (Gillooly and Zenil-Ferguson, 2014). Nonetheless, the most simple and reliable marker for oxygen carrying capacity is obtained by determining haematocrit (Fig. 1). This blood marker is determined by centrifugation, which also enables determining leukocrit for the same blood sample as a measure of animals' immune status (see also technical note in Annex 2). The measurement of RBC dimensions in an animal can also provide information regarding its metabolic rate, exposure to contaminants and acclimatization to environmental changes (Llacuna et al., 1996; Gregory, 2001; Davis, 2008). In addition, RBC size is positively correlated with genome size (Gregory, 2001), which enables identifying polyploid individuals of amphibians and fish via blood cell sizing (Garcia-Abiado et al., 1999). In mammals, a reduction in RBC size is also associated with anaemia (Table 1). Even though RBC size can be determined either using blood films or a cell suspension (see Appendix D), the former underestimates the real size of RBC in vertebrates (Palacios et al., 1987).

In addition to haematocrit and leukocrit, plasma and serum (plasma without some clotting factors) can be collected after blood centrifugation (Fig. 1), with the exception of reptiles for which serum is hard to collect. Likewise, collecting the blood cell pellet can be recommended for genetic analyses to concentrate DNA, especially in taxa without nucleated RBCs, and for stable isotope analysis (see below). Although plasma and serum enable determining the same blood markers, it is advisable for some techniques to collect one or the other. For instance, antimicrobial activity values are higher when measured in plasma than in serum (Liebl et al., 2009), and the presence of anticoagulants in plasma may interfere with techniques of seroneutralization (Ehret et al., 2002). Either in plasma or serum, the use of commercial kits currently facilitates the determination of a wide range of metabolites (e.g. glucose, lactate) (Table 3). A less specific but informative measurement is also the comparison of protein profiles by using agarose gel electrophoresis (e.g. Deem et al., 2009). Precise protein detection can, however, be obtained by enzyme-linked immunoassays (ELISAs), although these assays may be constrained in some species by a lack of specificity in the available antibodies (Evans, 2008). When fresh blood can be processed immediately in a laboratory, total blood cell counts can be determined by using flow cytometry or the improved Neubauer chamber (see Pendl, 2013), although all automated counters should be calibrated, especially to obtain accurate data from non-mammalian blood samples (Uchiyama et al., 2005; Douglas et al., 2010). These automated counters also enable differentiating certain blood cell abnormalities (e.g. micronuclei, Barata et al., 2010) but they still cannot detect the wide range of abnormalities that can be identified via the examination of blood smears (see ecotoxicology section).

More sophisticated techniques brought from the cell biology discipline can be applied for assessing specific aspects of immunity and cell integrity (reviewed by Boughton et al., 2011; Demas et al., 2011). In addition, blood samples preserved for traditional genetic analyses are suitable for the application of epigenetic techniques such as the luminometric methylation assay to determine global DNA methylation (Head et al., 2014). DNA methylation is one of the currently best understood mechanisms of epigenetic inheritance, and may explain how environmental factors, including contaminants can have lasting and even multigenerational effects on health in wildlife (Head, 2014). In humans, DNA methylation levels in WBCs are found to be correlated with individuals' susceptibility to disease (Terry et al., 2011) and pollutant concentrations in blood, including metals (Chanda et al., 2006) and persistent organic pollutants (Rusiecki et al., 2008). Yet little is still known about the consistency of these patterns, including natural differences in DNA methylation between blood cell types, and how all this research applies to wildlife. Most field researchers will, however, be unlikely to have access to these techniques due to logistic or financial constraints. Nonetheless, this gateway to cell biology and cutting-edge genetic studies using blood cells is another example of the versatile nature of blood samples to acquire in-depth knowledge of the toxic effects of contaminants and/or the immune response across vertebrates without euthanasia (Evans, 2008; Thrusfield, 2013).

3. Application of blood markers in ecology

Underlying drivers of animals' cost–benefit decisions broaden the scope of many studies in ecology (McNamara and Houston, 1996). One measure of costs is to determine changes in the nutritional status of animals facing biotic (e.g. predation) and abiotic (e.g. environmental change) challenges (McNamara and Houston, 1996). Glycogen and lipids are the main sources of energy stored in animals but in conditions of extremely high energy demands muscle tissue may also be used as energy source. Using a battery of 10 blood markers, Arizmendi-Mejía et al. (2013) reported a peak in creatinine kinase in the plasma of individuals of the seabird Cory's shearwater (*Calonectris diomedea*) during the pre-breeding period, indicating that muscle was degraded as an energy source for recovery from migration and preparation for breeding. Another example of trade-off associated with reproduction is the cost of development and maintenance of sexual ornamentation. Figuerola et al. (1999) reported that the size of yellow feathered areas in Cirl Bunting (*Emberiza cirlus*) males is related to the absolute number of WBCs and to the proportion of heterophils. Likewise, Polo-Cavia et al. (2013) demonstrated that head coloration in red-eared slider (*Trachemys scripta elegans*) reflects its health status using the heterophil (or neutrophil)/lymphocyte ratio. Further, van de Crommenacker et al. (2011) showed a direct link between habitat quality and the oxidative stress experienced by individuals of the endangered Seychelles Warbler (*Acrocephalus sechellensis*). Finally, work conducted in humans and birds also shows that leukocyte profiles can help predict an individual's future performance and viability (Davis et al., 2008).

In addition to trophic studies and determining individuals' body condition, animal movements can also be traced via blood markers such as fatty acid (FA) profiles and stable isotope analyses (SIA) (Rubenstein and Hobson, 2004; Tierney et al., 2008; Fig. 2A). The usefulness of these markers relies on their predicted deposition into consumers' tissue, providing insights into the prey and feeding ground used by an animal (Ramos and González-Solís, 2012). FA profiles have recently been incorporated into conservation science, with the additional advantage of being less influenced by geographical changes in baseline levels than SIA (Ramos and González-Solís, 2012). For SIA, the low lipid content of blood does not interfere in carbon and nitrogen isotope readings (Bearhop et al., 2000). An additional advantage is that blood components differ in isotopic turn-over rate (see blood constituent turn-over above) so a single sample can reflect changes in animals' diet over time (Ramos and González-Solís, 2012). Other uses of blood samples in ecology are the application of genetics for determining the gender in birds without sexual dimorphism (Jensen et al., 2003) and the genetic structure of populations, including paternity studies (Schmoll et al., 2009; Hu et al., 2011) and the invasion history of introduced species (Alda et al., 2013). Further, blood samples can be used to determine the rates of cellular senescence through the measurement of telomere lengths (Reichert et al., 2014), and the quantification of reproductive hormones to assess the breeding condition (Schultner et al., 2013).

4. The use of blood markers in environmental toxicology

The function of blood as a vehicle for transportation and distribution of pollutants following uptake makes blood suitable for screening contaminants in vertebrates. Some pollutants, such as lead, mercury or polycyclic aromatic hydrocarbons, show certain bioaccumulation in blood, faithfully reflecting the environmental concentrations (Pérez et al., 2008; Mieiro et al., 2009). Frequently, the external concentrations of these and other pollutants are positively related to blood levels, though generally lower than those found in the liver (the main tissue

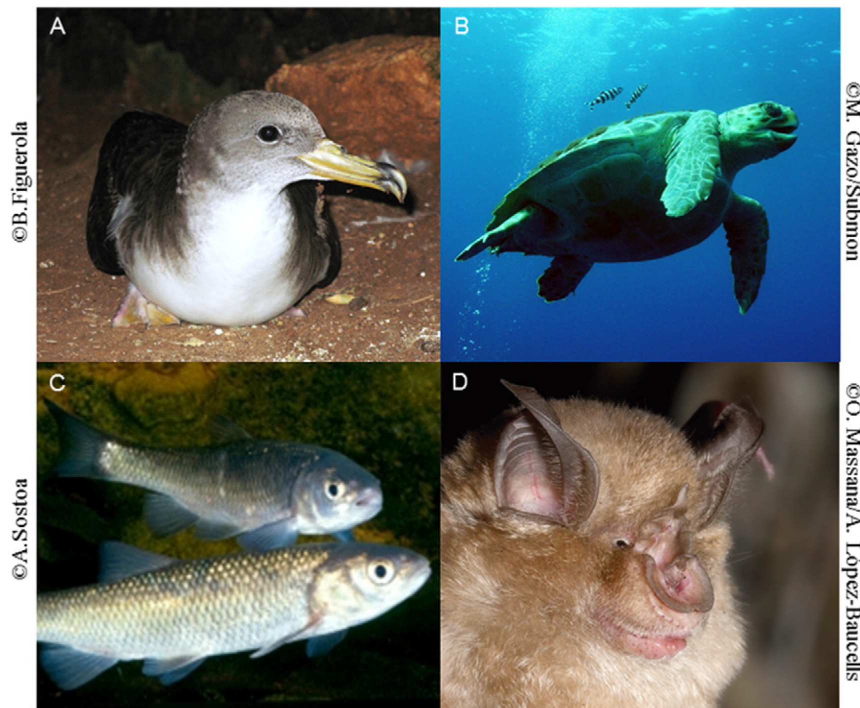

Fig. 2. Blood sample uses in studies of ecology, ecotoxicology and veterinary in wildlife. (A) The diet, foraging strategies and migratory pathways of many bird species (including the Cory's Shearwater *Calonectris diomedea*) can be determined by specific stable isotope analysis (Ramos and González-Solís, 2012); (B) the determination of pollutant concentration in endangered vertebrates, such as the Loggerhead sea turtle *Caretta caretta*, is used in monitoring studies for environmental risk assessment (Keller et al., 2004); (C) the presence of carcinogenic compounds in waters is evaluated by examining the blood cells of aquatic species, such as the Ebro chub *Squalius laietanus* (Maceda-Veiga et al., 2013); and (D) the spread of infectious agents with zoonotic risk can be determined by screening the blood of wild animals, such as the Greater horseshoe bat *Rhinolophus ferrumequinum* with serological and molecular techniques (Serra-Cobo et al., 2013).

depository) and fat (Henriksen et al., 1998; Fig. 2B). As blood is involved in the inter-tissue redistribution of contaminants, it can be particularly recommendable for monitoring pollution events in species with high mobility or in migratory stages (Pérez et al., 2008; Roscales et al., 2010). During migration, individuals mobilise energy stored in tissues where pollutants are also retained, thereby making blood suitable for detecting past pollution events (Roscales et al., 2010). Thus, blood samples can inform the bioaccumulation risk of certain pollutants in a given sentinel species and then such risk can be inferred for other wildlife taxa and humans over different spatial scales but especially for those living mainly in the same area.

For assessing the effects of pollutants, blood samples can indicate a direct impact upon the blood constituents and on systemic responses that are translated into plasmatic alterations (Evans, 2008). Regarding the effects on blood cells, the earliest responses include cellular adaptations to pollutant-associated overproduction of reactive oxygen species (ROS). Blood cells are amongst the first cells to suffer toxic effects and RBCs, in particular, are considered a major site for ROS production due to their role in the oxygen transport via haemoglobin (Ruas et al., 2008). Different enzymatic and non-enzymatic antioxidants can counteract ROS and some are used as oxidative stress markers in blood samples such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) activities and total glutathione (GSHt) (Guilherme et al., 2010). When inefficiently neutralized, peroxides and free radicals can damage cells, including proteins, lipids, DNA and RNA (Muniz et al., 2008). For instance, pollutants oxidise the long chain $n-3$ polyunsaturated fatty acids present in blood cell membranes (Nagasaka et al., 2004). Hence, lipid peroxidation in RBCs (and less frequently in plasma) is another common indicator of oxidative stress that can be measured in blood samples. A final indication of cytotoxic effects is an increase in the occurrence of dead RBCs in blood smears (Fig. 3). In this regard, the frequency of smudge cells indicates a pathological condition in humans typified by a high cellular fragility (see Johansson et al., 2010 for lymphocytes). However, smudge cells generally appear as undifferentiated cells on blood smears, i.e. without any identifiable cytoplasmic membrane or nuclear structure (Fig. 3), so we recommend not to include them in studies on the relative proportion of degenerated RBCs to avoid misleading conclusions.

For the assessment of DNA integrity, blood cells are consistently used as a mirror of damage in other body cells, especially in non-mammalian vertebrates (Barata et al., 2010; Fig. 2C, but see Frenzilli et al., 2009). Such damage can be detected by the quantification of an array of nuclear abnormalities in RBCs (Table 1; Fig. 3), but by far the most adopted and complementary assays are the comet assay and micronuclei (MN)/erythrocytic nuclear abnormalities (ENA) tests. Whilst the former detects early signs of damage that can be subjected to a repair process, ENA/MN tests signal chromosome breakage or loss and mitotic spindle apparatus dysfunction, which are hardly reparable lesions (Cabarcas-Montalvo et al., 2012; Guilherme et al., 2014). Highly informative but rarely used in ecotoxicology is the detection of ROS-induced DNA damage in blood cells indirectly by a sophisticated comet assay or directly by quantification of nucleotide-derivate in plasma (Oliveira et al., 2010).

In biochemistry approaches, any increase in the activity of specific intracellular enzymes in plasma is always an indication of tissue damage (Table 3). For instance, an elevated transaminase activity in plasma is generally used to signal liver damage (but see Wagner and Wetzel, 2009 for reptiles). There are, however, some confounding diagnostics using enzymatic activities. For example, an increase in lactate dehydrogenase may be associated with RBC breakdown due to bad sample preservation, pollutant-induced haemolysis (both easily detected in blood films) and/or muscle degradation (Douglas et al., 2010; Pendl, 2013). More specific enzymatic responses, such as cholinesterase inhibition in plasma/serum, are, however, unequivocally associated with exposure to some families of pesticides as shown in birds (Oropesa et al., 2013) and reptiles (Sanchez et al., 1997). Determining reproductive hormone

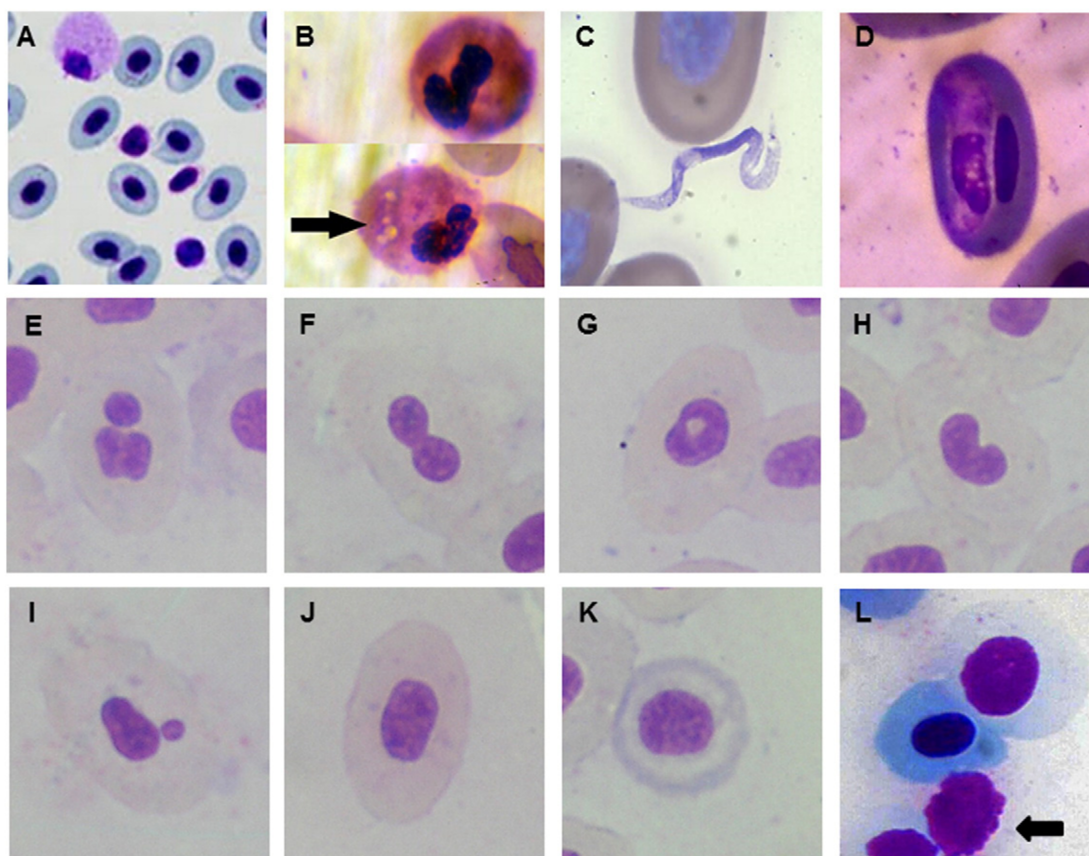

Fig. 3. Blood smears are the cheapest and simplest procedure to determine the health status of vertebrates via quantification of differential white blood cell counts (granulocytic white blood cell surrounded mostly by red blood cells, A) along with their degree of activation, such as the toxic granulation of heterophils (B), the detection of infectious agents, such as *Trypanosoma* spp. (C) and *Hepatozoon* spp. (D), and the determination of the percentage of red blood cells with lobed (E), segmented (F), vacuolated (G), kidney-shaped nuclei (H) and with micronuclei (I). A mature red blood cell (J) and an immature (K) red blood cell with normal nuclei are also shown along with a dead red blood cell (L) and smudge cell (see the arrow). *Trypanosoma* picture© A. Davis. See also Appendix E for complementary information.

levels also identifies unequivocally endocrine disruptions in vertebrates (Gerbron et al., 2014). These and other endocrine alterations can also influence carbohydrate and lipid metabolism (Schultner et al., 2013), depicted in altered glucose and lactate levels, as well as lipid profiles.

5. Infectious diseases and parasites

The examination of blood smears stained with conventional techniques (e.g. Diff Quick) can provide a first evidence of gross infection (e.g. an increase in numbers of neutrophils or heterophils) or parasitosis (e.g. increase in eosinophils number) (Tables 1 and 2). Distinguishing a stress response from that caused by disease and inflammation is indeed a major challenge of diagnostics based on differential WBC counts. As suggested by Davis et al. (2008), the two responses may be dissociated by examining the relative number of circulating monocytes and eosinophils as these cells do not increase in number under stress. A refinement in this diagnostic is also obtained by using cytochemical stains (see Martínez-Silvestre et al., 2005; Tavares-Dias, 2006), which highlight WBC traits (e.g. neutrophil or heterophil toxic granulation, Fig. 3 and Appendix E), associated with organism response to infections (Table 2). These results combined with an increase in total WBC count (see leukocrit in Appendix B) and/or a peak in the activity of some enzymes in plasma, such as aspartate-transaminase, will provide the final unequivocal but unspecific evidence of animal disease (Table 3). Some initiatives, such as the LYNX software for wild mammals, aid in disease diagnostics by integrating results from blood markers (Bennet et al., 1991). Some pathogens, such as haemoparasites and bacteria (septicaemia), can be directly detected on blood smears (Fig. 3 and Appendix E). However, identifying the target pathogen usually requires agglutination tests and/or the amplification of specific genes using Polymerase Chain Reaction (PCR) (Liebl et al., 2009; Maia et al., 2014; Fig. 2D). Nonetheless, the former method is limited by the set of antibodies available for wild taxa (Thrusfield, 2013).

Together with determining their pathogenicity, the detection of infectious agents in blood samples enables testing for ecological and evolutionary hypothesis, as well as increasing our understanding of their disease dynamics (e.g. Serra-Cobo et al., 2013; Arnal et al., 2014). In recent decades, monitoring the health status of wildlife has acquired a major interest after a growing number of emerging health issues affecting humans originated from wild animals such as West Nile virus, avian influenza and Ebola (e.g. Brook and Dobson, in press). This has raised an old paradigm renamed as 'One Health Initiative' (http://www.onehealthinitiative.com/), which aims to promote cross-disciplinary knowledge exchange amongst scientists to increase our understanding of health issues affecting wildlife, humans and environment. Besides its consequences for public health, such collaborative effort can improve our knowledge about how anthropogenic impacts affect biodiversity, including non-human infectious disease outbreaks and alterations in ecosystem services.

6. Conclusions

Haematology is still an opaque science for wildlife but promoting its standardised application in ecology and ecotoxicology is a challenge. This brief overview shows how traditional and cutting-edge techniques applied to blood samples contribute to increase the set of non-lethal procedures that researchers or resource managers can use in monitoring studies of wild vertebrates. Traditionally, veterinarians are responsible for

animal health diagnostic, but environmental health scientists and other practitioners can also raise health issues in wildlife following some basic diagnostic procedures. A more multidisciplinary framework in field studies is also essential for better understanding wildlife disease outbreaks and multi-trophic impacts on ecosystems. The perfect method to determine animal or ecosystem health does not exist, and it is the combination of indicators of impairment at different levels of organisation (e.g. community, population, and organism) that will give us the best diagnostic picture (Todgham and Stillman, 2013). Nonetheless, systematic blood analyses in wild vertebrates may serve as early indicators of population in trouble before the stress of a response significantly impacts reproduction and other measures of performance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.02.004.

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