Opinion

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New perspectives in tracing vector-borne interaction networks

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Disentangling trophic interaction networks in vector-borne systems has important implications in epidemiological and evolutionary studies. Molecular methods based on bloodmeal typing in vectors have been increasingly used to identify hosts. Although most molecular approaches benefit from good specificity and sensitivity, their temporal resolution is limited by the often rapid digestion of blood, and mixed bloodmeals still remain a challenge for bloodmeal identification in multi-host vector systems. Stable isotope analyses represent a novel complementary tool that can overcome some of these problems. The utility of these methods using examples from different vector-borne systems are discussed and the extents to which they are complementary and versatile are highlighted. There are excellent opportunities for progress in the study of vector-borne transmission networks resulting from the integration of both molecular and stable isotope approaches.

Bloodmeal identification studies in vector-borne systems

Approximately one-third of all infectious diseases are vector-borne, including many emerging pathogens of global human health and economic concern [1]. Vector-borne pathogens are often maintained in complex enzootic cycles involving multiple reservoir vertebrate hosts and competent arthropod vectors. These multi-host pathogens represent a great challenge to the study of emerging diseases because different host and vector combinations might contribute to disease dynamics in a distinct manner [2]. In vector-borne systems disease transmission occurs when a vector feeds on a susceptible host after having previously fed on an infected host. For some vector–pathogen systems both vertical (e.g. transovarial) and horizontal (e.g. venereal) transmission of pathogens can also occur directly between vectors. Transmission dynamics critically depend upon the capacity of the arthropod vector to acquire, maintain and transmit the pathogen (i.e. the vector competence) and the potential of the vertebrate host as a reservoir. Consequently, vector feeding ecology and host community composition can have dramatic consequences for understanding patterns of transmission in nature [3] and pathogen spillover to humans (Boxes 1–3) [4,5].

Hematophagous arthropods are vectors for many pathogens, and bloodmeal identification studies have shed light on vector feeding patterns and thus on the transmission potential of different vector groups [6]. Molecular methods based on bloodmeal typing in a wide range of blood-feeding

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Glossary

Discrimination or fractionation factor: is the difference in isotopic composition between an animal and its diet.

Enzyme-linked immunosorbent assay (ELISA): in the ELISA method enzymes are attached to either antigen or antibody. Upon linking the enzyme to the antigen or antibody, the complex retains both its immunological and enzymatic activities and in the final step a conjugate, or substrate, is added that the enzyme can convert to some detectable signal.

Hematophagous: refers to insect vectors feeding on blood (i.e. blood-sucking arthropods).

Heteroduplex analysis: heteroduplex analysis is based on the denaturation and reannealing of DNA strands to form homologous and heterologous double-stranded DNA molecules. In bloodmeal identification analyses, PCR products from the probe DNA (driver) are mixed with PCR products of tested DNA. During electrophoresis, the rate of migration of heteroduplex DNA through a polyacrylamide gel varies according to the three-dimensional conformations formed by mismatched base pairing between heterologous sequences and can be diagnostic for identifying DNA from different species.

Isotopic space: (isotopic niche) is the space occupied by a species in an n-dimensional isotopic niche, where each dimension corresponds to a particular isotope.

Mixing models: The isotopic composition of a mixed sample is determined by the isotopic signature and the contribution of the original sources by applying mass balance equations. Using the simplest forms it is usually only possible to determine $n + 1$ sources for n isotopes; thus, if data are available for two isotopes, for example carbon and nitrogen, it is possible to determine the contribution of three food sources if the isotopic value of all three food sources and the isotopic value of the target organism are known and distinct.

Multiplexed PCR: is a variant of the PCR test in which more than one target sequence is amplified using more than one pair of primers.

Parous rate: the proportion of individuals of the vector species that have already laid a batch of eggs.

PCR-restriction fragment length polymorphism (RFLP): following PCR amplification of a locus, the amplicon is treated with a restriction endonuclease. If the recognition site for this enzyme is present in the amplicon, two or more restriction fragments are generated. Thus, sequence variation between individuals at the recognition site(s) can be detected by electrophoresis.

Precipitin test: this test depends upon the interaction between a saline extract of the bloodmeal under examination and animal antiserum. A positive reaction is indicated by the formation of a white precipitate that results from the production of insoluble antigen–antibody complexes.

Reverse line-blot hybridization: this method consists of a single-run PCR amplification of a conserved gene by using non-degenerated primers, followed by a reverse line-blot hybridization assay. Amine-conjugated oligonucleotide probes are covalently coupled to a carboxylated nylon membrane and then biotin-labeled PCR products are hybridized to the probes. Hybridized PCR products are visualized colorimetrically by incubating the membrane with streptavidin-labeled peroxidase and a chemiluminescent substrate.

Sensitivity: in the considered case is the probability that a vector that fed on a given host will test positive for that host.

Specificity: here applied is the probability that a vector that has not fed on a given host will test negative for that host.

Trophic niche: is the space occupied by an organism within the environment in relation to its food and the consumers that prey upon it.

Box 1. Use of bloodmeal identification for understanding West Nile virus epidemics

West Nile virus (WNV) is an emerging disease in North America. Since its introduction in 1999 in New York it has extended throughout the continent causing more than a thousand deaths and several hundred thousand cases of illnesses in humans (CDC 2010, http://www.cdc.gov/ncidod/dvbid/westnile). Transmitted by mosquitoes, WNV has been detected in more than 300 species of birds, which are the main reservoirs, 30 mammals and several reptiles and amphibians [4]. Mammals are 'dead host ends' because virus viremias in the blood remain too low to infect mosquitoes. The use of molecular methods to identify the bloodmeal origin has allowed rapid progress to be made towards understanding the factors related to virus amplification in the wild and the causes of epidemics in humans.

In addition to the simple division of mosquitoes into ornithophilic and mammalophilic based on their preferences for these large groups of vertebrates, previous studies have identified great differences in the origin of the bloodmeal in the mosquitoes of a single species trapped in different localities (Figure I). The proportion of bloodmeals from humans reported in different studies ranged from 0% to 18%. Bloodmeal origin not only differs between localities but also from one season to another [5]. The consequences of these changes in vertebrate bloodmeal sources could be vital for understanding

transmission to humans. The bloodmeal composition of *Culex* mosquitoes was identified from five different localities [4]. Preferences of mosquitoes were not determined by the local abundance of vertebrate species and they focused most of their feeding on a small number of bird species. Combining feeding preferences with information regarding host competence for WNV, the relative reproductive ratio ($R_{0,rel}$) was calculated, which measures the changes in the pathogen reproductive ratio (R_0) due to mosquito feeding preferences and heterogeneity in vertebrate host competence. Values of $R_{0,rel}$ correlated well with the date of the first isolation of WNV in mosquitoes in 2004, thereby suggesting that WNV amplification was higher in those localities. Heterogeneity in the feeding preferences of mosquitoes has consequently emerged as an important factor in understanding WNV amplification. Interestingly, the feeding preferences of *Culex pipiens* and *Culex tarsalis* were not constant over time and changed between the end of summer and autumn [5]; as *Turdus migratorius*, the preferred host of mosquitoes, disperse from its breeding areas in California and Colorado, mosquitoes shifted their feeding preferences to humans. This diet shift occurred even when the overall abundance of birds increased. Changes in the diet of mosquitoes were the prelude to significant increases in the number of human cases of WNV infection reported in the study area.
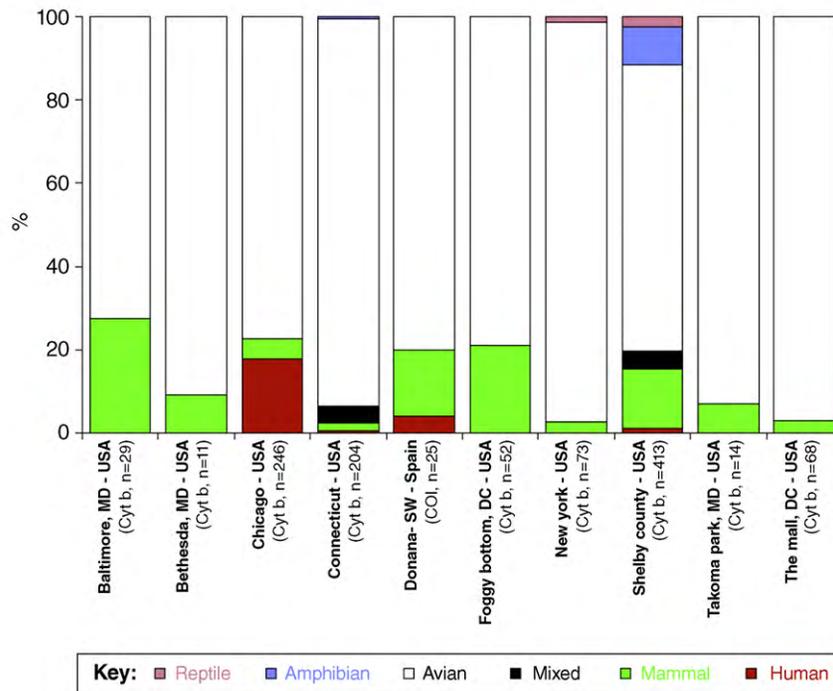

Figure I. Host feeding preferences in mosquito vectors. Percentage of meals from taxonomic groups of different hosts based on identification of *Culex pipiens* mosquito bloodmeals by PCR-based methods. The sample sizes of mosquito meals and the diagnostic markers employed are shown in parentheses. Source data are from [4,9,41–44]. The origin of vertebrate blood differs widely between species but even within species large local or seasonal differences occur. In the case of *C. pipiens*, strong differences have been found in the proportion of human derived bloodmeals (ranging from 0% to 18%) or the proportion of avian derived bloodmeals (69–97%), and consequently strong differences in the risk of transmission of pathogens from birds to humans could be expected.

arthropods have been increasingly used to identify hosts [7,8]. Compared with more traditional methods (e.g. serological tests such as the enzyme-linked immunosorbent assay, ELISA, and the precipitin test, [6]), PCR-based approaches considerably improve the performance of the tests; however, they still face several technical challenges in the case of vector-borne network studies (e.g. mixed bloodmeals and digestion status). Although there has been much research on mosquito transmission networks, little is known about the problems and limitations associated with

other vector pathosystems (e.g. involving fleas, ticks or lice). Although some of the novel DNA-based approaches can be applied to almost any vector arthropod [9], the time and laboratory costs are still high, particularly in the study of transmission networks of neglected diseases with no human health or economic interest and in areas with a great variety of potential hosts [10].

Naturally occurring stable isotopes are widely used in ecological studies of trophic food webs [11]. Although rarely used in host–parasite interaction studies [12], stable isotope

Box 2. SIA methodology

Natural abundance of stable isotopes

Isotopes are atoms whose nuclei contain the same number of protons but a different number of neutrons, and thus have a different atomic weight. Stable isotopes are energetically stable, occur naturally in the environment, are safe and non-radioactive and do not decay. These isotopes also vary in mass and in biologically important elements (e.g. C, H, N, O and S). Very small differences in mass cause subtle differences in isotope behavior during chemical reactions and diffusion. This process, known as isotopic fractionation, alters the ratio of heavy to light isotopes and can lead to slight variations in isotopic composition in nature.

Notation and terminology

Isotopic composition is usually expressed as delta (δ) values, that is the isotopic value of a sample in relation to an internationally accepted standard expressed as the deviation per parts per thousand from that standard: δ (‰) = $(R_X/R_{ST}) \times 1000$, where R is the respective isotope ratio of the heavy-to-light isotope (e.g. $^{13}C/^{12}C$ or $^{15}N/^{14}N$), R_X is the ratio in the sample and R_{ST} is the ratio in the standard.

The process of isotopic fractionation is quantified with fractionation or discrimination factors (α or Δ), which can be defined as the ratio of two isotope ratios (e.g. the isotopic ratio of a consumer and its diet). Fractionation factors and the isotopic composition of two substances are related as: $\alpha A - B = (1000 + \delta A)/(1000 + \delta B)$. For bloodmeal determination, A represents consumer tissue and B vertebrate host blood.

Stable isotope ratio spectrometry

Stable isotope ratios are measured using an isotope ratio mass spectrometer, which separates charged atoms or molecules on the basis of their mass-to-charge ratio. For more details on the different methodologies and applications see [45]. Sample processing is a crucial part of the whole analysis and very often an important source of error. The procedure depends on the physical form of the sample and the element to be measured. Usually, samples are collected, dried, finely ground and weighed into a small tin or silver cup (approximately 5×8 mm) for C, N and S, or O analysis, respectively. Over the past 20 years the cost and complexity of the SIA methodology have decreased dramatically, and nowadays SI methods require only minimal preparation after collection, resulting in samples that are easy and safe to dispatch.

SIA in food web studies

The utility of stable isotopes in food web studies is based on the fact that different dietary items often have different isotopic signatures, which in turn are reflected in the tissues of consumers (i.e. 'you are what you eat'). The process of ingestion, digestion and assimilation by consumers is associated with a shift in isotopic ratios and the magnitude of these 'shifts' is referred to as fractionation or discrimination factors, or trophic enrichment. As far as the different blood sources have different isotopic signatures, this would enable the identification of the vertebrate source of the bloodmeal in the vector (Figure I).
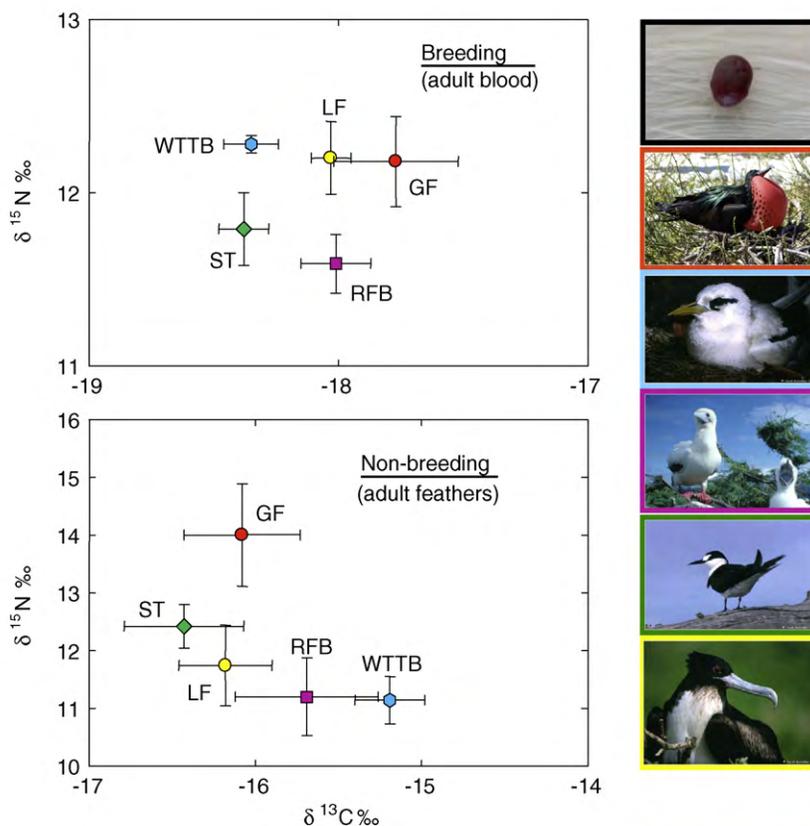
TRENDS in Parasitology

Figure I. SIA analysis in multi-host vector systems. This is an example of the application of SIA in the study of a hypothetical multi-host system food web involving the tick *Ornithodoros capensis* and several sympatric seabird host species. Note that the stable carbon and nitrogen isotope analyses reveal clear isotopic structure in the seabird community, thereby allowing species to be identified, although there are slight variations depending on the type of host tissue analyzed. Values are means (±SD) of the carbon and nitrogen isotope values in the blood (upper panel) and feathers (lower panel) of adult seabirds from Europa Island (Indian Ocean). Figure adapted from [46]. Pictures by P. Calabuig, J. González-Solís and taken from Wikimedia commons.

Abbreviations: GF, great frigatebird; LF, lesser frigatebird; RFB, red-footed booby; ST, sooty tern; WTTB, white-tailed tropicbird.

Box 3. SIA applications in studying vector diets

The main shortcoming of PCR-based methods for bloodmeal typing relates to the rapid digestion of blood in the vector. SIA, however, is not sensitive to DNA degradation and thus is not affected as much by this problem. Rasgon [13] was first to apply stable isotope analysis to the identification of bloodmeals. By using proof-of-principle experiments, Rasgon demonstrated that SIA could identify the mosquito bloodmeals after complete digestion. After blood-feeding, the mosquito *Aedes albopictus* assimilated diagnostic carbon (C) and nitrogen (N) stable isotope signatures from their vertebrate hosts that could be accurately identified 1 week after feeding, even once the entire bloodmeal had been digested. Figure I summarizes these results; on the basis of the C/N isotope ratio, Rasgon was able to distinguish between blood-fed and unfed mosquitoes, and differences in the δN values were diagnostic for identifying the host type. Related to this experiment, one application of this is the estimation of parous rates in the vector. The vector survival rate is a very important factor that determines vectorial capacity [47]. In the case of mosquitoes, the traditional method employed involved the dissection of females and an examination of ovarian tracheoles to determine whether the mosquitoes had ever bred (nuliparous) or had already laid one or several batches of eggs (parous) [48]. Fed and unfed mosquitoes could be identified even after all bloodmeal had been digested [13]. Consequently, using SIA, it could be possible to estimate from a single-pooled sample the parous rates of species that need to have digested a bloodmeal to produce eggs.

The utility of SIA in bloodmeal identification studies has also been demonstrated under natural conditions. Previous field studies have suggested that the exploitation of alternative hosts by prairie dog fleas (*Oropsylla hirsuta*) favored the spread of the causative agent of plague, *Yersinia pestis*, in North America. SI analyses were applied to the vector-borne system of plague [14,40]; by examining N isotope values of fleas collected from grasshopper mice, both prairie dogs and grasshopper mice were found to contribute equally to flea bloodmeal, whereas isotopic signatures of fleas collected from

burrows indicated that they contained diminishing bloodmeals from prairie dogs [14]. In another study, a species-specific PCR assay was applied to this system and revealed that 57% of *O. hirsuta* collected from mice contained mouse DNA. Nevertheless, no host DNA could be identified in the remaining 43% of fleas or from over 200 fleas collected from burrows. This was probably because fleas digest blood from prairie dogs more quickly than blood from mice. Degradation of host DNA in prairie dog fleas was concluded to preclude its identification using conventional PCR assays (unless fleas are collected soon after feeding) [40].
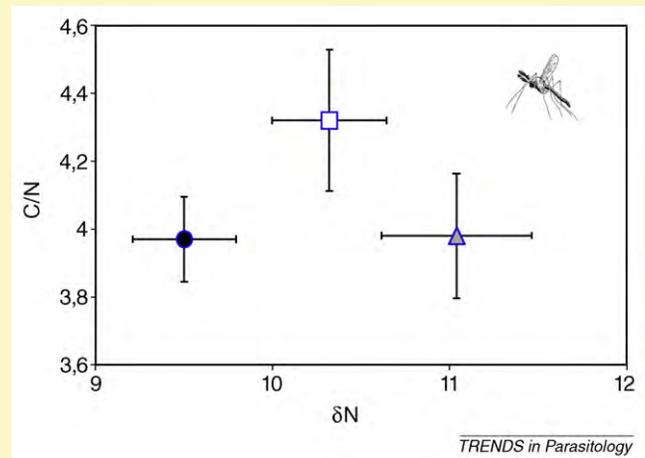

Figure I. SIA identification of mosquito bloodmeals. Plot of C/N ratio versus δN for feeding treatments; $n = 10$ for mosquitoes per treatment. Plots are sample means $\pm 95\%$ confidence intervals. The different host types are indicated: unfed (white square), chicken-fed (black circle) and human-fed (grey triangle) mosquitoes. Figure from [13].

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analyses (SIA) could become a useful tool for bloodmeal identification [13,14]. In terms of usefulness and application to the identification of bloodmeals, SIA is in its infancy and still has important shortcomings that are common to food web analyses. Nevertheless, recent studies have shown that it can be an excellent complementary tool for overcoming some of the limitations inherent in current DNA-based approaches.

Uses and caveats of molecular methods in the study of vector diets

A variety of different DNA-based methods have been employed for typing arthropod bloodmeals and usually rely on sequencing or amplifying a target locus of the host (i.e. mitochondrial, ribosomal or nuclear loci) (reviewed in [8]). A major challenge common to all blood identification studies is posed by the fact that many hematophagous arthropods are generalist feeders which exploit a wide range of vertebrate host species, including birds, mammals, amphibians and reptiles [15]. Several wide-spectrum methods such as heteroduplex analysis [16], reverse-line blotting [17], PCR-RFLP [18], and multiplexed PCR [19] are widely used given that they increase sensitivity, thereby allowing detection of multiple hosts; although, for correct assignment of host species, such methods require known standards for recognition. Indeed, the specificity of these tests largely depends upon a comprehensive reference database against which unknown samples can be compared. Fortunately, blood-feeding studies can make

use of public databases such as GenBank [20] or recent global sequencing initiatives such as DNA barcoding (http://www.boldsystems.org) [21,22]. Nevertheless, the coverage provided by public molecular reference databases is still limited.

Successful detection by PCR-based methods largely depends on the presence of a minimum amount of undigested blood. Thus, there are important time constraints when samples are obtained, given that host DNA is gradually digested by the vector. Indeed, the time window and thus the ability to identify hosts via bloodmeal analyses depends upon the arthropod vector in question and environmental conditions [23]. In this regard, mosquitoes are particularly problematic. Most trapping methods fail to attract females that have recently fed [24], whereas other techniques such as gravid traps tend to attract engorged females that might have already digested the bloodmeal. Depending on the ambient temperature and the vector and the host species involved, successful DNA amplifications can only usually be obtained within a period of up to 36–72 h after the mosquito has ingested the blood [25–27]. By contrast, ticks differ from other blood-feeding arthropods given that their bloodmeals are large and slowly digested (up to 280 days post-engorgement [28]). Nevertheless, the success of PCR-based methods with ticks varies considerably between studies and between seasons within the same study [29–31]. Another important factor affecting PCR success is the length of the amplified fragment. Short amplicons are more likely to amplify in degraded DNA.

This can introduce a serious bias when using primers designed to deal with different groups of hosts but that might widely differ in the length of the amplified product [19].

In multi-host vectors, the arthropod bloodmeal can contain mixed blood from a variable number of often unrelated host species; yet, determining the role of the different reservoir hosts in the community is crucial for an understanding of disease dynamics. Mixed bloodmeals represent a particular caveat for bloodmeal identification studies and, given dominant template bias and variation in host probe sensitivity, some host species might be more detectable than others by PCR-based methods [32]. This can cause a serious bias when, for example, trying to estimate the risk posed by a disease vector in infecting a particular host (e.g. in the case of humans and mosquitoes [26]). Cloning could allow the different species in mixed bloodmeals to be identified, although this strategy is expensive and time-consuming. In well-characterized vector systems, an alternative strategy is the post-hoc analysis of mixed chromatographs [9], although data interpretation is not easy, especially if fragments from more than two host species are amplified.

### SIA cross-application to the study of vector diets: 'you are what you eat'

SIA (primarily carbon, reported as $\delta^{13}C$, and nitrogen, $\delta^{15}N$) are among the most widely applied and powerful tools for characterizing food webs [11]. This approach is based on the fact that natural stable–isotope ratios are transformed from the source to the consumer in a predictable manner (Box 2) [33]. The isotopic composition of parasite tissues should be a function of the isotopic signature of each host species and of the relative proportions of each host species assimilated. In addition, owing to the isotopic fractionation associated with converting host tissue into parasite tissue, a trophic shift in isotope values is expected between a consumer and its diet (typically 2–3% greater) [34]. The isotope ratios can then be measured in an arthropod vector and inferences regarding its diet and feeding behavior can be made. In the case of arthropods, the stable isotope approach has been successfully applied to several groups including flies, ants and aquatic insects [35].

Given these examples, one clear advantage of SIA that arises is its versatility and cross-application to different vector–host systems. The use of several stable isotopes such as nitrogen, carbon, hydrogen, oxygen and sulfur, or specific compounds such as amino acids and fatty acids, can make it easier to differentiate between potential hosts in diverse communities [33,36]. As with PCR, the comparative analysis of the isotopic signatures of vector and hosts, that is the determination of discrimination factors (see Glossary), requires the development of a library of isotopic signatures of the possible hosts in an area. This is the main limitation of SIA regarding bloodmeal identification. Undeniably, as the number of potential hosts increases, the possibilities of clearly separating the host species in the isotopic space are considerably reduced. Once the isotopic space of the different hosts has been characterized, the trophic niche of the vector can be estimated. A variety of

statistical approaches commonly used in community trophic ecology (e.g. mixing models) can be applied to predict the percentage contribution from each host [34]. If a vector has fed on numerous individual hosts, the mixed bloodmeal will have a differential isotopic composition. The greater the isotopic difference between hosts, the less uncertainty associated with the calculation of each contribution. But the degree of isotopic distinctiveness and thus the level of resolution will rely on the characteristics of the biological system and accordingly should be better evaluated on a case-by-case basis. Recent studies used mixing models to estimate the proportions of two possible blood sources in fed fleas collected from rodents and equivalent contributions from both main and alternative hosts (58% and 42%, respectively) were found, highlighting the potential utility of SIA in the quantitative and qualitative estimation of vector feeding patterns [14]. Nevertheless, individual variation in isotope ratios could be important (e.g. in relation to the size, age, developmental stage and sex of individuals), as could be environmental variation caused by seasonal or local changes in isotope baseline levels [37]. Owing to variable tissue turnover and discrimination factors, different tissues might have distinct isotopic signatures [38], and in the case of hematophagous vectors host blood is the best possible reference tissue to use.

In addition to considering isotopic enrichment between the vector and its host, the critical starting point of SIA in multi-host vectors is related to the existence of enough isotopic discrimination between possible hosts. These differences could be taxon-specific (e.g. mammals or birds) or depend upon the ecology of the host related to, for instance, differences in their trophic ecology or their spatial segregation [37]. Despite great differences in isotopic signatures among major taxonomic groups [38], the resolution with SIA is significantly reduced when compared to molecular methods. In this regard, it is clear that SIA will perform best when there are a limited number of possible host species, but when combined with PCR approaches the number of possible applications can be multiplied. Owing to the intrinsic multidimensionality of SI fractionation factors (e.g. individual, physiologic and spatial variation), SIA could provide further insights beyond taxonomic distinctiveness (see Figure I in Box 2).

Despite the intrinsic limitations of SIA, the main strength of this approach is that it represents a novel and complementary tool to PCR-based methods and partially overcomes some of the limitations associated with bloodmeal analysis. SIA could be more sensitive than PCR because it has the potential to allow one to distinguish between potential hosts even after all the blood has been digested (Box 3), that is the stable isotope signatures of the blood are not affected by DNA degradation. As the bloodmeal is digested, it is assimilated into vector tissues and an isotopic shift occurs, enabling us to gain knowledge about previous feeding events. Previous research on the vector-borne plague system is a good example (Box 3) [14]. Moreover, in the case of fleas, previous research has shown specific patterns in flea digestion rates depending on the host species, individual status and even host sex [39].
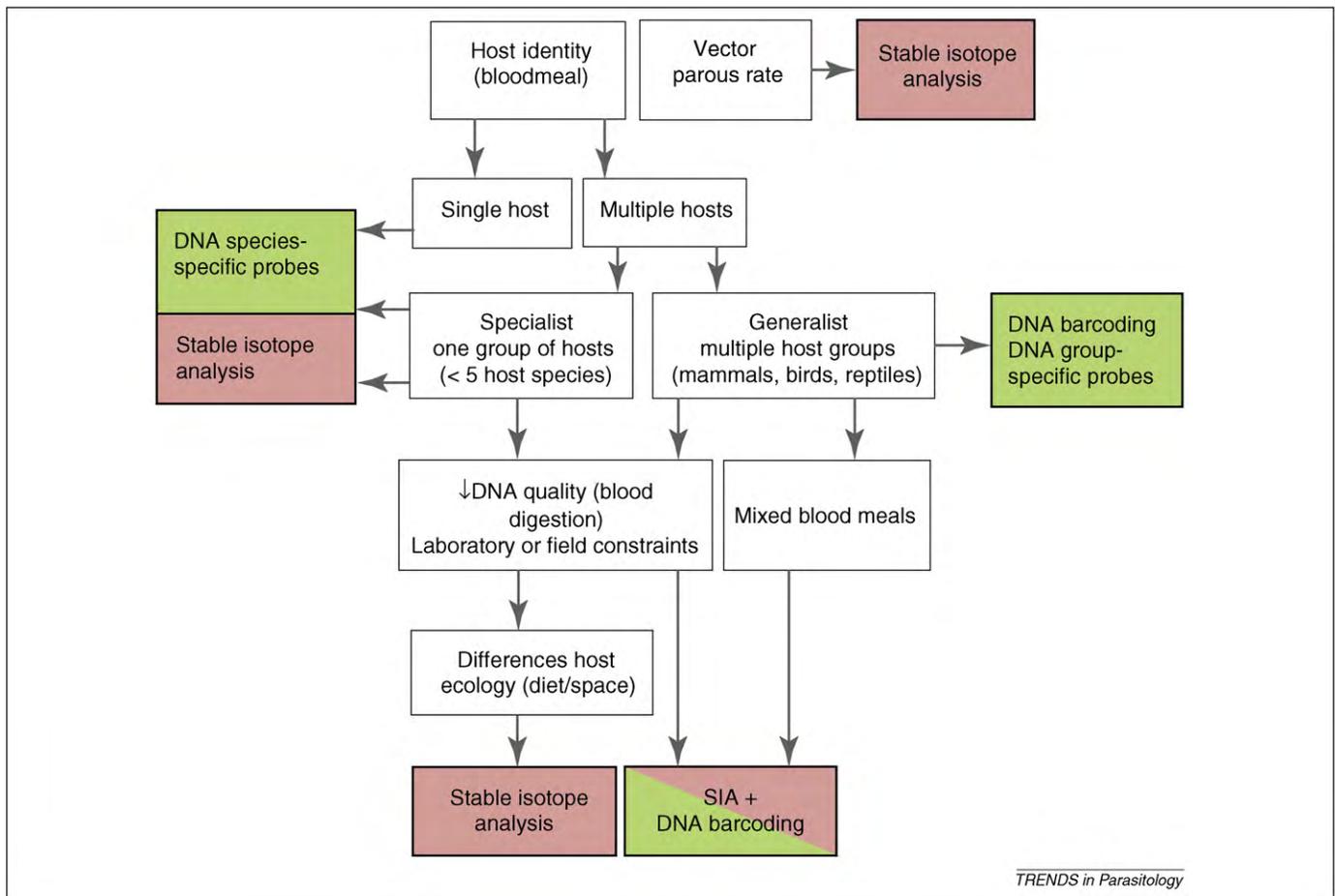
Figure 1. Genetic versus isotopic approaches for bloodmeal analysis. Flow chart showing the most recommendable approaches, molecular or isotopic, which will depend on the characteristics of the biological system (i.e. vector specialism), and the time window for the identification of the bloodmeal sources. Although molecular approaches are the method of choice in most situations, stable isotope analyses (SIA) are particularly suitable in situations of DNA degradation either due to bad laboratory or field conditions or because of digestion by the arthropod vector. In the case of multiple hosts species and mixed bloodmeals, the level of resolution of SIA will rely on the isotopic distinctiveness among potential hosts either linked to differences in their diets or habitat use. Another potential and yet unexplored application of SIA is the determination of the parous rate of the vector.

Consequently, if used alone, PCR-based methods could provide a biased picture of vector feeding patterns and ultimately of the transmission dynamics in this system.

The determination of parous rates in the vector population is a straightforward but an as yet unexplored use of SIA. Previous experiments suggest that it might be possible to estimate the parous rate as the proportion of fed and unfed mosquitoes from the SIA of a single pooled sample (Box 3) [13].

Overall, genetic and isotopic technologies could well provide complementary information into the feeding ecology and vectorial capacity of disease vectors when used conjointly (Figure 1). Whereas molecular methods provide greater specificity and accordingly can be used to obtain specific information on the source of recent bloodmeals, SIA can provide complementary information on older and time-integrated feeding events, which owing to DNA degradation might be difficult to obtain from PCR approaches [13,40]. In terms of multi-host vectors and for the problem of mixed bloodmeals, the contribution of SIA is probably limited to simple host systems (Box 2), although in these systems it could be useful to decipher the relative contribution of different hosts to the bloodmeal [14].

Concluding remarks and future directions

Both DNA-based approaches and SIA have important applications in health and conservation management in the field of emerging diseases; however, in many vector-borne systems identifying the roles of multiple reservoir hosts and competent vectors remains a major challenge in blood identification studies. In this regard, the combination of biogeochemical and molecular markers can overcome the limitations inherent to each technique and help us gain new and clearer insights into the ecology of vector-borne diseases. Whereas molecular methods provide greater specificity in terms of host species identification, SIA can elucidate cryptic feeding relationships, which could be difficult using DNA–PCR approaches. Despite this potential, the cross-application of techniques such as SIA remains largely unexplored in the study of host–parasite interactions, particularly in vector-borne systems. Food web analysis methods continue to develop and could be applied in many novel ways to host–parasite studies and disease epidemiology. Over the coming years, lower costs and reduced complexity together with new technological advances will probably enhance the potential of these indirect approaches and increase their contribution to epidemiological research.

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