Host identification in unfed ticks from stable isotope compositions (

Host identification in unfed ticks from stable isotope compositions ($\delta^{13}C$ and $\delta^{15}N$)

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Abstract

Determination of the ratios of natural stable isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in unfed *Ixodes ricinus* nymphs and adults that in their previous stage fed on captive wild rodents (*Apodemus sylvaticus*, *Myodes glareolus*), wild birds (*Parus major, Cyanistes caeruleus*) or domestic ruminants (*Ovis aries*, *Bos taurus*) demonstrated that it is possible to identify each host category with confidence. Firstly, the tick–blood spacing, which is the difference between values obtained from ticks and the blood of hosts that they had fed on in the previous stage were consistent (152 spacings investigated from 15 host individuals in total). Secondly, potential confounding factors (tick age and sex) did not affect the discriminatory power of the isotope patterns, nor did different rearing conditions (room temperature versus 4°C) or the duration of development (maximum 430 days). The findings that the tick-blood isotope spacings, across a diverse range of hosts, were similar and predictable, and that confounders had little or no effect on this, strongly promote the usage of the isotope approach. As each of the host categories have different roles in the population dynamics of *I. ricinus* and in tick-borne pathogen ecology, the presented method has great potential for the elucidation of tick and tick-borne pathogen ecology in the field.

Key words. Isotope Ratio, Spectrometry, *Ixodes ricinus*, stable isotopes, tick-borne diseases

Running head. Isotopic tick host identification

Introduction

The analysis of natural stable isotope compositions of organisms has been widely used to identify cryptic trophic interactions in food webs (Newton, 2016). Recent studies revealed its potential to unravel complex interactions between a free-living parasite and its hosts (Stapp and Salkeld, 2009; Schmidt et al., 2011; Demopoulos and Sikkel, 2015). Isotope ratio analyses could discriminate between nutrient sources (i.e. hosts), which the parasite has consumed, even a long time after feeding.

The analysis of isotopic compositions in parasites can therefore potentially make a significant contribution to the elucidation of local transmission cycles of vector-borne diseases. The method makes it possible for hosts that are sources of pathogens and nutrients for ticks to be identified, which is essential information needed for targeted prevention measures. However, without meticulous methodological evaluation with regard to underlying assumptions of isotopic interpretations (e.g. known tick–host isotope spacing) and the natural life cycle and stressors that affect the vector's development and thus possibly isotope composition, the utility of stable isotope analysis remains uncertain (Schmidt et al., 2011; Jenkins et al., 2018).

Here we advance the development of the method for identification of members of the host community from which the developmental stages of *Ixodes ricinus* L. ticks obtained their blood meals. As is the case in all ixodid tick species, *I. ricinus* feeds once per parasitic stage (larva, nymph, adult) for several days, after which they detach and develop to the next stage (nymph, adult, egg). It is therefore possible to identify a prior host by analysis of the unfed nymph or adult resulting respectively from the moult of the fed larva or nymph. The identification of tick hosts is an important component of the analysis of tick population dynamics and of the ecology of the pathogens that ticks transmit. Although it

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is possible to obtain some information by collecting feeding ticks directly from the hosts, unfed exophilic ixodid ticks are much more readily available to the researcher, because they can be collected from the vegetation in large numbers by blanket-dragging or flagging. Such field-caught unfed ticks contain small remnants of the blood meal obtained by the previous stage and several attempts have been made to identify hosts by detecting their DNA (Kirstein and Gray, 1996; Pichon et al., 2005; Humair et al., 2007; Moran-Cadenas et al., 2007; Allan et al., 2010) or proteins (Vennestrom and Jensen, 2007; Wickramasekara et al., 2008; Laskay et al., 2012; Onder et al., 2013; Onder et al., 2014) in these blood-meal remnants. These methods have met with some success, but they are limited by their complexity, expense and dwindling sensitivity as the blood meal degrades in the tick gut.

An alternative approach is based on the measurement of ratios of naturally occurring stable isotopes (e.g. $^{15}N/^{14}N$, $^{13}C/^{12}C$) incorporated into animal tissues from the nutrient elements in an animal's food. It takes advantage of the fact that the isotopic composition of a host is transferred to a blood-feeding arthropod via the host's blood. In two proof-of-concept studies using a small set of laboratory animal species, it was shown that the host isotopic signature of the blood is replicated in unfed stages of the ticks, *Ixodes ricinus* (Schmidt et al., 2011) and *Amblyomma americanum* (Hamer et al., 2015).

In the present study, *I. ricinus* larvae and nymphs were fed on hosts representing natural ecological categories (small mammals, ruminants and birds) under controlled conditions, with the objective of determining the consistency of isotopic compositions in ticks and their host blood (tick–blood spacing) within particular host categories. Additionally, possible confounding effects of tick age, sex and developmental conditions (rearing temperature, developmental diapause) were investigated. This research will enable the identification of the host category fed on by a previous tick stage of collected exophilic specimens.

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Materials and Methods

Study design

The rodents involved in the study were maintained on commercial laboratory rodent diets, the cattle on hay cubes and milk replacement (PANTO Kälbergold), and the sheep on hay cubes and sheep concentrates (IBEKA Schäferstolz). The birds received a diet of mealworms and seeds. *Ixodes ricinus* were fed under controlled laboratory conditions (19-20°C) on rodents (larvae on two *Apodemus sylvaticus* and *Myodes glareolus* individuals), birds (larvae on three *Cyanistes caeruleus* and three *Parus major* individuals; nymphs on six additional *Parus major* individuals), ruminants (nymphs on two *Bos taurus* and four *Ovis aries* individuals).

Engorged ticks were kept under laboratory conditions (approx. 20°C, 90 RH, long-day photoperiod) for further development. A subset of unfed ticks from each host species were sampled (n = 17*3 nymphs; n = 20 adults) and frozen (-20°C), three months after the moulting of larvae and nymphs into nymphs and adults, respectively. Nymphs were pooled (n=3 per sample) to attain sufficient sample mass for the instrument setup used at the time of this study (see Schmidt et al., 2001); adult ticks were sexed and analyzed individually. Some ticks from birds were maintained under short-day photoperiods and subsequently entered developmental diapause. A subset of the resulting adults was sampled three (n = 6 with diapause) and six months after engorgement (n = 12 with diapause, n = 5 without diapause).

To quantify possible effects of tick age and temperature, developing ticks were kept at either room temperature (20°C) or refrigeration temperature (4ºC) simulating summer and winter conditions, respectively, and sampled at approximately 3-month intervals for up to 15 months (n = 58*3 nymphs, n = 40 adults in total). Host blood was collected in

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capillaries and sampled twice (no samples of six additional *Parus major*). A droplet of blood was obtained by puncturing a vein with needle or lancet (ulnar vein in birds, submandibular vein in rodents, ear vein in ruminants). Whole ticks were freeze-dried and weighed in 5 x 3 mm tin capsules. Blood and tick C and N stable isotope compositions (expressed as conventional delta values per mil, δ ‰) were determined at the NERC Life Science Mass Spectrometry Facility, East Kilbride, Scotland, by continuous flow elemental analysis – isotope ratio mass spectrometry (CF–EA–IRMS), using a Costech ECS 4010 elemental analyser interfaced with a ThermoFisher Scientific Delta XPPlus IRMS. Samples were analyzed blind by the EA–IRMS operator, i.e. without knowledge of the tick treatment or host species, because tin-wrapped samples were supplied in multiwell plates with unidentifiable sample codes.

In total, the following numbers of ticks were analyzed per host: *Apodemus sylvaticus* (n = 28*3 nymphs), *Myodes glareolus* (n = 16*3 nymphs), *Parus major* (n = 17*3 nymphs and 23 adults), *Cyanistes caeruleus* (n = 14*3 nymphs), *Bos taurus* (n = 32 adults), and *Ovis aries* (n = 22 adults).

Statistical analysis

General linear mixed effects models (normally distributed residuals) were fitted for comparisons between the host categories and host species (nested within categories), taking into account the correlation of the repeated measurements from the same bird individual and the hierarchical structure of random effects (Molenberghs and Verbeke, 2005). Multiple-group discriminant analyses (Sharma, 1996) were performed in order to (1) identify the variables that discriminate best between the vertebrate classes, (2) to compose an index, (i.e. new variables) that represent the differences between the groups,

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to (3) classify future observations into one of the groups, and (4) to evaluate the membership by means of resubstitution technique. The objective of this method is finding a linear combination of the variables (i.e. index), such that the scores of the subjects in the same group are very similar (minimizing the within sum of squares) and the scores of the subjects from different groups are quite different (maximizing the between sum of squares). In each analysis it was assumed that the probability of any given observation belonging to any of the groups is the same. Therefore, the pooled within covariance matrices will be used in the discriminant function. Data management and statistical analyses were performed using SAS v 9.2 (SAS Institute, Cary, North Carolina, USA).

Results

Inter- and intra-vertebrate class analyses 90 days after moulting

Ninety days after the fed tick larvae had moulted into nymphs, the tick–blood spacing for $\delta^{15}N$ differed statistically among host categories, i.e. ruminant, rodent or bird (interaction category*tick–blood: $F_{1,25} = 17.23$; $P < 0.01$), however the ranges of the absolute spacings were very limited (range spacing: 3.70 ± 0.09‰ (birds) to 4.08 ± 0.08‰ (rodents)). For $\delta^{13}C$, the spacing did not differ significantly between categories (category*tick–blood: $F_{1,25.1} = 4.15$; $P = 0.054$) and on average was not shown to be statistically different from zero (estimate: -0.05 ± 0.09‰; $F_{1,26.2} = 0.32$; $P = 0.58$). In the nymph-exposed hosts, the adult-blood spacing was very similar for rodents, ruminants and avian host categories; no statistical differences were found among host categories in either $\delta^{15}N$-spacing or $\delta^{13}C$-spacing (all P's > 0.05; see Table 1 for estimates). For both the nymphs (resulting from fed larvae) and adults (resulting from fed nymphs) the tick-blood spacing for $\delta^{13}C$ was

more variable than for $\delta^{15}N$ (Table 1 and Fig. 1). Nevertheless, in the discriminant analyses all ticks could be correctly assigned to vertebrate host categories of origin, as was the case of the blood samples (all error count estimates equal 0.0 %; Appendix 1). Moreover, the host species within each vertebrate category could be identified with low to reasonable error rates by means of discriminant functions calculated from the blood's isotope composition (error counts ruminants: Cow vs. Sheep = 0%; birds: great tit vs. blue tit = 25%; wood mouse vs. bank vole = 30%) (Appendix 2). Differences in $\delta^{15}N$ tick-blood spacing were found neither between the bird members (species*tick–blood: $F_{1,13} = 0.31$; $P = 0.59$), nor between rodent members (species*tick–blood: $F_{1,10} = 3.33$; $P = 0.10$). In contrast, $\delta^{13}C$-spacing was higher in great tits than in blue tits (estimate Δ *great tit – blue tit*: 0.81 ± 0.16 ‰; species*tick–blood: $F_{1,13} = 26.7$; $P = 0.0002$) and higher in wood mice than in bank voles (estimate Δ *wood mouse – bank vole*: 0.49 ± 0.19 ‰; species*tick–blood: $F_{1,10} = 6.63$; $P = 0.027$) which is also shown in Figure 1. The $\delta^{15}N$-spacings in adult ticks from ruminants significantly differed between host species ($F_{1,15.2} = 17.10$; $P = 0.0009$), although the difference was small compared to the average spacing (estimate Δ *cow – sheep*: 0.50 ± 0.12 ‰; 11 % of average spacing in ruminant). The $\delta^{13}C$-spacing in adult ticks did not differ at all between the sheep and cattle (species*tick–blood: $F_{1,14.1} = 0.02$; $P = 0.89$).

[Table 1.]

Effects of confounders

Confounder effects were almost absent; when statistically significant they were small. Therefore, isotope tick-blood spacing remained relatively constant, and ticks could be

classified with high certainty in the inter-host category level (all error count estimates < 0.04%) and still relatively high certainty on the intra-host category level (error count estimates < 0.20 %; Appendix 1).

In the batches of tick individuals that were kept for a longer time under different temperature conditions (4°C vs. room temperature), stable isotope compositions enabled us to distinguish the vertebrate classes on which they have fed, both in adults and nymphal ticks (Figure 1). While $\delta^{15}N$ was not significantly affected by the time since moult (sampling up to 15 months) nor by temperature, $\delta^{13}C$ slightly increased with time (nymphs: 0.0019 $\pm$ 0.0006 ‰/days, $F_{1,65.5} = 11.05$; adults: 0.0019 $\pm$ 0.0003 ‰/days; $F_{1,71.3} = 24.97$, P-values < 0.001). In addition, in the nymphs that moulted from engorged larvae, $\delta^{13}C$ was on average higher when ticks were kept at room temperature (estimate Δ *cold-room*: -0.28 ± 0.12 ‰; $F_{1,63.2} = 5.68$; P = 0.02). In none of the analyses did gender explain a substantial part of the variation.

[Table 2.]

[Fig. 1.]

Some of the engorged nymphs that fed on great tits underwent developmental diapause before moulting into adults (n = 18 individuals). In comparison with the $\delta^{15}N$ values in adult ticks that experienced a regular development ($\delta^{15}N$: 7.74 ± 0.46‰), they had similar $\delta^{15}N$ values when sampled six months after moulting (7.83 $\pm$ 0.29‰; T-value= 0.17, df = 25, P = 0.87, n = 12 individuals). $\delta^{15}N$ values in diapaused ticks were lower when the sampling occurred three months after moulting ($\delta^{15}N$ ‰ = 5.80 $\pm$ 0.41; T-value= 1,93, df = 25, P = 0.0043, n = 6 ticks belonging to unknown bird individual). $\delta^{13}C$ values in diapaused ticks were significantly lower than the bird-fed ticks that did not undergo

diapause (T-value=160.54, df = 25, P < 0.0001), but were similar to the change over time in other host types. Spacing in diapaused adult ticks are not further analyzed, since information on blood isotopes was unavailable for the six additional birds on which they fed.

Discussion

The most important findings of this study are that tick-blood isotope spacings, across a diverse range of hosts, were similar and predictable, and that confounding factors had little or no effect on this. Confirmation of these two fundamental assumptions will enable researchers to apply isotope ratio measurements to assign field-caught ticks to hosts (or at least trophic host groups) they fed on. The present study used captive hosts, however numerous studies have shown that wild, sympatric host species in the same habitat are often segregated by dietary requirements and hence isotope composition of their blood (e.g. Yi et al., 2006; Baltensperger et al., 2015). The observed tick-host spacing values are well within the range reported from most other food webs (Caut et al., 2009).

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Isotopic spacing and confounders

This study has validated for the first time critical pre-conditions for assigning hosts to field-caught ticks. Firstly, the isotopic spacing between tick and host blood showed remarkably little variation among vertebrate categories, especially for $\delta^{15}N$. $\delta^{13}C$, on the other hand, seems to be more variable, and hence is likely to have less discriminatory power to identify the host on which the ticks have fed. Age and gender had limited effects on the isotopic compositions of ticks. In contrast to the earlier proof-of-concept study

(Schmidt et al., 2011) tick age was of little influence, possibly because the sampling of the ticks in the present study commenced after the excretion of blood remnants (occurring in the first few weeks after the moult) while in the first study the sampling occurred immediately after the moult. The sample strategy in the present study was more realistic because in the wild, ticks do not start questing until the cuticle has hardened after a few weeks (Balashov, 1972).

Possible effects of developmental diapause were investigated in bird-fed ticks only. Ticks that were sampled six months after moulting, showed very similar $\delta^{15}N$ values with ticks that did not undergo diapause. The $\delta^{15}N$ values in diapaused ticks sampled three months after moulting were unexpectedly lower than at six months, but this was probably due to the combination of the larger individual variation in base-line blood values in the omnivorous *Parus major* (difference max. - min. $\delta^{15}N$: 2.25) and sampling bias (all ticks originated from a single bird with low $\delta^{15}N$ tick values). Similar to *Cyanistes caeruleus* (difference: 1.70) the among-individual $\delta^{15}N$ variation was larger than in the other, herbivorous host species (differences < 0.66).

Overall, the observed lack of impact by confounders is pivotal for the application of isotope compositions, as the local populations of questing ticks at a given moment in time contain mixtures of individuals from different cohorts, with varying successes in host finding due to stochastic processes (Gray, 1991; Randolph et al., 2002). The predictable spacings, do not seem to have any of the unusual or unexpected spacings reported for other generalist parasites (Nachev et al. 2017).

Classification and protocol

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In the present controlled experiment, host categories could be differentiated reliably with isotope techniques in unfed ticks, as shown by the low classification error rates. Because the members within these host categories (i.e. host species) generally have overlapping dietary requirements, there is a high likelihood of overlap in the isotope signal as well, which is demonstrated here by higher classification error rates (both in blood tissue and ticks). It is important to note that all host animals in the present study were held in captivity and the feed stuffs used may not represent food choices in wild populations. However, it is highly likely that, in a given habitat, vertebrate host categories with known differences in dietary habits (e.g. herbivorous deer versus omnivorous woodmice versus insectivorous birds) will have different isotopic compositions (Yi et al., 2006; Baltensperger et al., 2015).

After sampling and analysis of the blood of locally available vertebrates, researchers will have obtained solid information that can be used to predict the position of the questing ticks within the isotope ratio space (Figure 1), and thus to deduce a tick's blood sources (i.e. vertebrate hosts on which they have fed). We provide linear discriminant functions (Appendix 1 and 2) that can be used for validation in the field. However, as host diets may depend on habitat types and regions, we recommend that local prior screenings should be conducted. Furthermore, it is obvious that the discriminant functions for the differentiation of host categories are different from those required to classify the members within these categories. We therefore suggest a stepwise approach in which, after mapping the isotope ratios of blood samples of the host community, discriminant functions are first created that maximize the differentiation of ecological host categories. The subsequent discriminant analyses will be designed to categorize the species within these categories (Appendix 2). In the long-term, an alternative to the sampling of local hosts for determination of tick feeding patterns would be a multi-factorial model, built on data of given habitats in which host sampling has occurred, enabling us to predict local

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blood isotope ratios for host categories and thus their utilization by the local tick population. However, before this can be achieved it is necessary to extend laboratory-based studies to the field in order to determine the variation in differentiated host categories. Eventually it should be possible to determine tick utilization of a host population by analysis of individual sampled ticks (including nymphs), particularly since advances in micro-analytical dual IRMS techniques now allow as little as a few µg of C and N to be analysed (Langel and Dyckmans, 2014). Methodical advances in non-destructive DNA extraction from small arthropods (e.g. Sakamoto and Gotoh, 2017) may even make it possible in the future to use a single tick to combine DNA analysis of host-specific pathogen strains and/or blood remnants with isotopic analysis of host origin.

In conclusion, this study has shown that it is possible to identify natural hosts of *I. ricinus* by analyzing unfed ticks, along with the blood of available hosts, for natural stable isotope ratios. Although specificity of blood remnant identification via molecular methods is high, failure of detection is considerable, particularly for aged ticks that have completely digested host DNA (Kirstein and Gray, 1996; Pichon et al., 2005; Humair et al., 2007; Moran-Cadenas et al., 2007; Allan et al., 2010). The proposed method therefore offers an alternative, and could be used in a complementary manner with existing molecular screenings. We believe that the described approach can be applied to other host/tick species interactions (including reptiles and humans), as long as dietary habits among hosts are dissimilar and individuals feed on a single host per developmental stage.

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Table 1. Tick–blood isotope spacing for $\delta^{13}C$ and $\delta^{15}N$ in adult and nymphal *Ixodes ricinus* fed under controlled conditions on ruminants, rodents, and birds. Ticks were sampled 90 days after the moult. Means are presented with one standard error. Figures in bold are means for a vertebrate category.

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		Adult tick-blood		Nymphal tick-blood	
Vertebrate category	Host – a,b	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	$\delta^{13}C$ ‰	$\delta^{15}N$‰
Ruminants		***0.56 ± 0.04***	***4.63 ± 0.14***		
	Cattle (*B. taurus*) - 2,2	0.57 ± 0.09	4.33 ± 0.19		
	Sheep (*O. aries*) - 3,2	0.56 ± 0.04	4.82 ± 0.03		
Rodents				***0.15 ± 0.20***	***4.08 ± 0.08***
	Woodmouse (*A. sylvaticus*) - 2,2	N.D.	N.D.	0.40 ± 0.03	3.97 ± 0.11
	Bank vole (*M. glareolus*) - 2,2	N.D.	N.D.	-0.10 ± 0.33	4.19 ± 0.02
Birds				***-0.13 ± 0.20***	***3.70 ± 0.09***
	Blue tit (*C. caeruleus*) - 3,2	N.D.	N.D.	-0.53 ± 0.19	3.73 ± 0.04
	Great tit (*P. major*) - 3,1	N.D.	N.D.	0.28 ± 0.07	3.66 ± 0.19
	Non-diapause (day 181*)	1.13 ± 0.04	4.54 ± 0.63		

a: # host individuals,

b: # tick samples per host (1 tick per sample in adults, 3 ticks per sample in nymphs)

* 5 ticks were sampled 6 months after moult; blood isotope values used from larva-exposed birds

N.D.: No Data

Table 2. Analysis of confounder-effects on the $\delta^{13}C$ and $\delta^{15}N$ values in ticks fed under controlled conditions on ruminants, rodents, and birds. Ninety-day-old ticks were kept either under cold (4°C) or room temperature (approx. 20°C) for several months (maximum 430 days). The gender of the ticks was recorded for adult ticks (i.e. fed nymphs that moulted into adults). Means with one standard error are presented for fixed effect (F-tests) that significantly differ from zero. All random-effects (Wald-tests) were kept in the models, in order to respect the hierarchical structure in the data.

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	Nymphs		Adults	
Fixed effects	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	$\delta^{13}C$ ‰	$\delta^{15}N$‰
Time since moult (/day)	$F_{1,65.6} = 11.05$*	$F_{1,62.4} = 0.04$	$F_{1,71.3} = 24.97$***	$F_{1,43.4} = 3.86$
	0.0019 ± 0.0006		**0.0019 ± 0.0003**	
Temperature	$F_{1,63.2} = 5.68$*	$F_{1,61.1} = 0.24$	$F_{1,72.6} = 3.86$	$F_{1,57} = 0.00$
Δ cold – room	**-0.28 ± 0.12**			
Gender	N.D.	N.D.	$F_{1,68.6} = 0.14$	$F_{1,72} = 1.31$
Time*Temperature	$F_{1,65.1} = 0.04$	$F_{1,61.8} = 0.00$	$F_{1,40.2} = 0.95$	$F_{1,50.8} = 0.08$
Time* Gender	N.D.	N.D.	$F_{1,66.6} = 0.52$	$F_{1,65} = 0.62$
Temperature* Gender	N.D.	N.D.	$F_{1,67.2} = 2.50$	$F_{1,67} = 0.00$
Time*Temperature* Gender	N.D.	N.D.	$F_{1,64} = 0.31$	$F_{1,67} = 0.07$
Random effects				
Species	Z = 1.22	Z =1.21	Z =0.79	Z =1.0
	4.94 ± 4.06	6.62 ± 5.47	0.019 ± 0.02	2.77 ± 2.76
Individual (Species)	Z =1.16	Z = 1.74*	Z =-0.09	Z =-4.93***
	0.07 ± 0.06	**0.23 ± 0.13**	-0.0006 ± 0.0070	-0.042 ± 0.009
Residuals	Z =5.32***	Z =5.49***	Z =5.93***	Z =5.99***
	0.20 ± 0.04	**0.22 ± 0.04**	**0.11 ± 0.02**	**0.54 ± 0.09**

Significance levels:

*, **, ***: *P*-values respectively < 0,05; < 0,01; < 0,001

N.D.: No Data

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