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Effects of anticoagulants on stable-isotope values ($\delta^{13}C$ and $\delta^{15}N$) of shark blood components

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Abstract

The effects of anticoagulant EDTA and sodium heparin (SH) on stable carbon $\delta^{13}C$ and nitrogen $\delta^{15}N$ isotopic values of red blood cells (RBC) and blood plasma in juvenile blacktip reef sharks *Carcharhinus melanopterus* were analysed. Plasma preserved with anticoagulants was not isotopically distinct from plasma stored in no-additive control tubes but RBC $\delta^{15}N$ values exhibited small enrichments when preserved with EDTA and SH. Results suggest EDTA and SH are viable anticoagulants for stable isotopic analyses of blood fractions but further studies are advised to validate results.

KEYWORDS
blood plasma, blood preservatives, *Carcharhinus melanopterus*, $\delta^{13}C$, $\delta^{15}N$, red blood cells (RBC)

Investigating the trophic ecology of marine predators, such as sharks is crucial for understanding their ecological roles and importance (Heithaus *et al.*, 2008; Roff *et al.*, 2016; Speed *et al.*, 2012). Using naturally occurring carbon and nitrogen stable isotopes as chemical tracers provides the opportunity to examine the trophic ecology of sharks and other taxa over various time scales (Matich, Ault, *et al.*, 2017). Stable isotope ratios of carbon 13C:12C ($\delta^{13}C$) and nitrogen 15 N:14 N ($\delta^{15}N$) can depict the food webs in which consumers are foraging and their relative trophic position (Hobson, 1999), respectively. Stable-isotope analysis (SIA) can also provide further insight into ontogenetic shifts in trophic interactions, long and short-term movements and individual foraging specialisations (Kiszka *et al.*, 2015; Matich *et al.*, 2011; Matich, Kiszka, *et al.*, 2017; Papastamatiou *et al.*, 2010; Speed *et al.*, 2012), but interpretation of isotopic data must be made cautiously (Thomson *et al.*, 2018)

Depending on the study questions and species of interest, a variety of body tissues are used for SIA in sharks (Hussey *et al.*, 2012). Metabolically inert (*e.g.*, bones, fin) or active (*e.g.*, liver, muscle, blood) tissues can be sampled independently, or in combination they allow for the investigation of potential temporal dietary changes (Bearhop *et al.*, 2004; Matich *et al.*, 2019; Matich & Heithaus, 2014). The use of blood fractions, specifically red blood cells (RBC) and blood plasma, has increased over recent years (Vander-Zanden *et al.*, 2015). RBC and plasma are especially useful to assess trophic positions and foraging behaviours of juvenile sharks (Hussey *et al.*, 2017; Kinney *et al.*, 2011; Matich *et al.*, 2015; Matich, Kiszka, *et al.*, 2017), because they incorporate trophic interactions over different time scales and can be collected non-lethally. RBC stable-isotope values reflect energy sources (*e.g.*, foraging and maternal provisions) over extended periods (multiple months), while plasma stable-isotope values represent more recent trophic interactions (weeks to months; McMeans *et al.*, 2009; Vaudo *et al.*, 2010; Matich *et al.*, 2019).

Despite the advantages of using blood for SIA, one of the major limitations of its use is its rapid coagulation. To obtain accurate isotope values for RBC and plasma, they need to be separated immediately but remote and logistically challenging field conditions may

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impede such rapid centrifugation. Therefore, alternative solutions that prevent tissue degradation are needed. Blood preservatives, such as anticoagulants, provide a potential solution. Anticoagulants inhibit the coagulation process and extend the time before centrifugation is needed. Common preservatives for blood collection and storage include EDTA and sodium heparin (SH). EDTA prevents the coagulation cascade through its binding ability of calcium and magnesium as a chelator (Banfi *et al*., 2007; Rand *et al*., 1996) and SH stimulates the production of antithrombin III to inactivate thrombin (Shuman & Majerus, 1976).

In order to correctly interpret stable-isotope values in blood tissues of young sharks, it is critical to know if blood anticoagulants bias these values. Recent investigations in leopard sharks *Triakis semifasciata* (Girard 1855) showed that RBC and plasma collected in tubes coated with lithium heparin (an anticoagulant) were not isotopically distinct from blood collected in no-additive tubes (Kim & Koch, 2012). In contrast, studies on birds and sea turtles revealed that stable-isotopic signatures are influenced by anticoagulants and the magnitude and nature of shifts in isotopic values varied among taxa, blood fractions and anticoagulants (Bugoni *et al*., 2008; Lemons *et al*., 2012). Consequently, by investigating the effects of a different assortment of anticoagulants on blood components in another species of sharks, we aim to extend the currently limited knowledge of blood preservative effects on isotopic values in shark blood. In this study, we investigated whether EDTA and SH preservation modified $\delta^{13}C$ and $\delta^{15}N$ values in RBC and plasma of blacktip reef sharks *Carcharhinus melanopterus* (Quoy & Gaimard 1824).

Juvenile sharks were captured with gillnets (20.0 m × 1.5 m, 5.0 cm mesh) at St. Joseph Atoll, Republic of Seychelles (05° 26′ S, 53° 20′ E) from November to December 2014. After the insertion of PIT tags (Biomark; www.biomark.com) and total length measurements (L_T), blood was collected from the caudal vein using 5 ml syringes (BD Plastipak; www.bd.com) with 18 gauge needles (BD PrecisionGlide). Each 5 ml blood sample was split into three tubes: 2 ml in EDTA coated tubes (BD), 2 ml in SH coated tubes (BD) and 1 ml in no-additive (control) tubes. To avoid coagulation, the latter sample was immediately spun at 1500 *g* by a hand-powered centrifuge (Hettich; www.hettich.ch) for 30 s and resulting blood components (RBC and plasma) were separated and kept in no-additive tubes. Together with the EDTA and SH treated samples, all tubes were kept on ice for a maximum duration of 6 h.

At the laboratory, blood samples preserved in anticoagulants were spun with a mini-centrifuge (Mini Fuge, STARLAB; www.stalabgroup.com) at 2000 *g* for 1 min and then frozen with control samples at −18°C. After 7 days, blood samples were placed in a drying oven (60°C) for 72 h and homogenised with a mortar and pestle. Subsequently, to determine the abundances of carbon (^{13}C:^{12}C) and nitrogen (^{15}N:^{14}N), 500–800 μg of the homogenised powder was loaded into tin capsules and analysed by a continuous-flow isotope-ratio mass spectrometer (Finnigan Delta C EA-IRMS (with temperature conversion element analyser; TC-EA), Thermo Fisher Scientific; www.thermofisher.com) with bovine liver, International Atomic Energy Authority (I-AEA)-N-1, I-AEA-C-6 and glycine used as standards.

TABLE 1 Mean (± SD) of $\delta^{13}C$, $\delta^{15}N$ and C:N values for control (no-additive) and blood components treated with anticoagulants (EDTA and SH) in *Carcharhinus melanopterus* captured at St. Joseph Atoll, Seychelles

Tissue	*n*	$\delta^{13}C_{CON}$	$\delta^{13}C_{EDTA}$	$\delta^{13}C_{SH}$	$\delta^{15}N_{CON}$	$\delta^{15}N_{EDTA}$	$\delta^{15}N_{SH}$	$C:N_{CON}$	$C:N_{EDTA}$	$C:N_{SH}$
RBC	11	−9.04 ± 0.84	−9.15 ± 0.86	−9.08 ± 0.83	10.89 ± 0.45	10.88 ± 0.6	10.77 ± 0.59	2.83 ± 0.08	2.79 ± 0.13	2.79 ± 0.06
Plasma	11	−8.95 ± 0.75	−8.87 ± 0.83	−9.20 ± 0.82	10.84 ± 0.75	11.35 ± 0.78	10.63 ± 0.74	1.80 ± 0.12	1.76 ± 0.05	1.83 ± 0.33

Abbreviations: CON, control; EDTA, ethylenediamine tetraacetic acid; *n*, sample size; RBC, red blood cells; SH, sodium heparin.

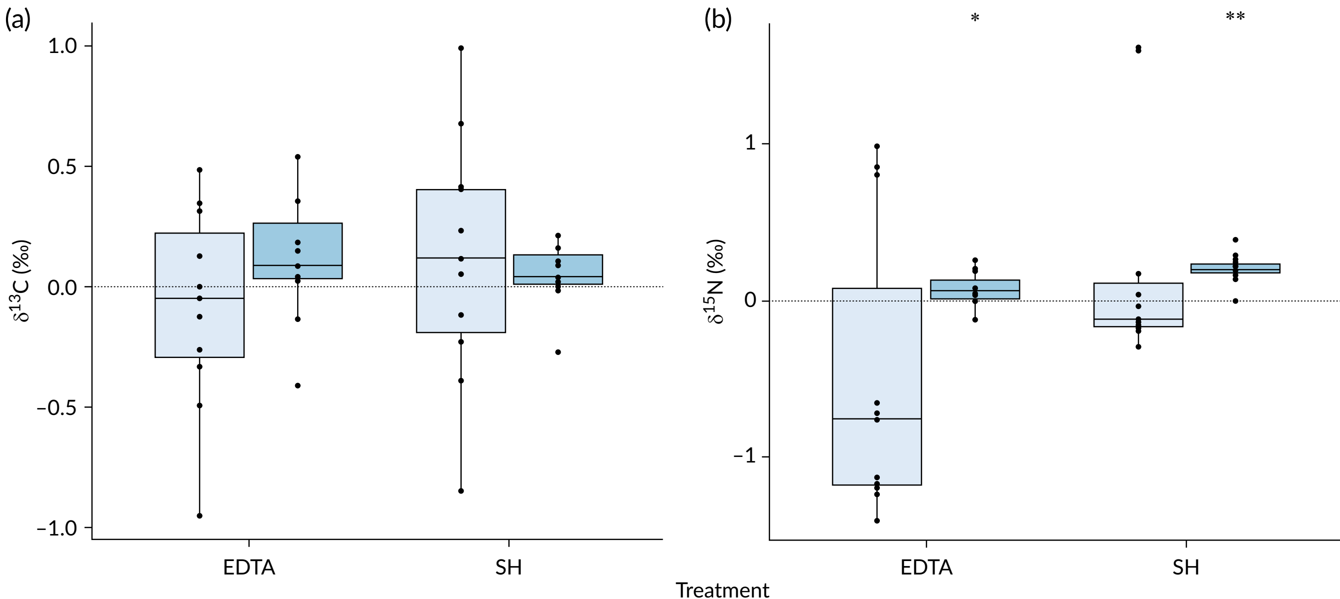

FIGURE 1 Boxplots ((——), median; (□), inter-quartile range; (|), 95% range) of individual and mean differences in δ^{13}C and δ^{15}N values between no-additive control and treated samples for all blood components in *Carcharhinus melanopterus* captured at St. Joseph Atoll, Seychelles. (………), Zero difference between control and treated samples. Significant difference from control: *, $P < 0.05$; **, $P < 0.001$ (see Table 2 for details). EDTA, ethylene diaminetetraaceitic acid; RBC, red blood cells; SH, sodium heparin. Tissue: (▭) Plasma, (▭) RBC

TABLE 2 Statistical results from paired analyses between control (no-additive) and blood components treated with EDTA or SH in *Carcharhinus melanopterus* captured at St. Joseph Atoll, Seychelles

Test	Tissue	Parameter	Mean difference	*n*	*t*-Test	Two-tailed *P*-value	Comments
Control *v*. EDTA	RBC	δ^{13}C	0.1118	11	1.4347	> 0.05	
		δ^{15}N	0.0873	11	2.6966	**< 0.05***	
		C:N	0.0349	11	43 (v)	> 0.05	Wilcoxon test
	Plasma	δ^{13}C	−0.0854	11	−0.6803	> 0.05	
		δ^{15}N	−0.5064	11	−1.8205	> 0.05	
		C:N	0.0305	11	1.0121	> 0.05	
Control *v*. SH	RBC	δ^{13}C	0.0445	11	1.1276	> 0.05	
		δ^{15}N	0.2018	11	7.3136	**< 0.001***	
		C:N	0.0369	11	1.0102	> 0.05	
	Plasma	δ^{13}C	0.2482	11	0.7918	> 0.05	
		δ^{15}N	0.2164	11	1.007	> 0.05	
		C:N	−0.0367	11	43 (v)	> 0.05	Wilcoxon test

Abbreviations: EDTA, ethylenediamine tetraaceitic acid; *n*, sample size; RBC, red blood cells; SH, sodium heparin.
* denotes significant difference.

Variation among laboratory standard for samples were 0.15‰ for δ^{13}C and < 0.10‰ for δ^{15}N. Lipid extractions were not conducted, because C:N for RBC (mean ± SD = 2.8 ± 0.08) and plasma (1.8 ± 0.03) were below those recommended for extraction or mathematical correction (Hussey *et al*., 2012).

Where parametric assumptions were met (assessed with Shapiro–Wilk tests), we used paired *t*-test to determine whether δ^{13}C, δ^{15}N and C:N isotopic values from treated samples (EDTA and SH) were statistically different from non-additive control samples. Where assumptions were not met for δ^{13}C, δ^{15}N and C: N, we applied a Wilcoxon signed rank test to compare differences between treated and non-additive control samples. Also, power analyses were run for comparisons. All the statistical analyses were performed in R (version 3.5.3; R Core Team 2017; www.r-project.org) within the RStudio interface 1.0.153 (RStudio Team, 2016) and the level of statistical significance α was set at 0.05.

Eleven juvenile *C. melanopterus* ranging in from 54.6–78.0 cm L_T (mean ± SD = 62.8 ± 7.7 cm L_T) were collected (mean δ^{13}C, δ^{15}N and C:N values in Table 1). For RBC, there was no significant difference in δ^{13}C values nor in C:N values between no-additive control and treated samples. The difference in mean δ^{15}N values for no-additive control and EDTA and SH treated samples was small (0.0873‰ and

0.2018‰, respectively), but significant (Figure 1 and Table 2). No-additive plasma δ^{13}C, δ^{15}N, or C:N were not different from treated plasma. Mean differences in δ^{13}C and δ^{15}N were considerably higher in plasma than RBC, except for δ^{13}C between control and EDTA treated samples (mean difference – 0.0854‰; Table 2).

To our knowledge, the present study is the first characterisation of the effects of EDTA and SH on stable-isotope values in RBC and plasma in sharks. Our results revealed that RBC and plasma isotope values were highly similar in no-additive control samples and samples treated with anticoagulants. The only significant differences between control and treated samples were found in RBC δ^{15}N values treated with either EDTA or SH. Despite statistical significance, differences among treatments were small, particularly for EDTA (< 0.1 ‰; Table 2), suggesting minimal effects of these anticoagulants on isotopic values in blood components.

Overall, our study confirms and extends the findings provided by Kim and Koch (2012), where blood components treated with lithium heparin yielded accurate isotopic data for shark blood. This is a promising outcome for the increasing number of isotopic studies on blood components (Vander-Zanden *et al*., 2015), but interpretation must be made cautiously. Our limited sample size may have resulted in smaller treatment effects and mean differences between control and treated plasma samples showed high variability for both anticoagulants (Figure 1 and Table 2). This observation is further supported by low statistical power (0.2) and there remains a potential for type II error, particularly for plasma samples. Further research should therefore aim at larger sample sizes and potentially include multiple shark species in order to validate if results are comparable and applicable for isotopic studies on a wide range of sharks.

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AUTHOR CONTRIBUTIONS

O.C.W. designed and coordinated the study; O.C.W. collected field data; O.C.W. analysed the data and wrote the manuscript with support from J.J.K, P.M. and M.R.H.; all authors gave approval for publication. O.C.W. secured the funding to conduct this study.

ETHICS STATEMENT

All research on sharks at St. Joseph, Seychelles was approved by and conducted with the knowledge of Ministry of Environment, Energy and Climate Change, Seychelles. Animal handling and tagging methods were conducted in accordance with the approved guidelines of S. Planes by the Autorisation de pratiquer des expériences sur les animaux n° 006725 (1995) from the ministry of Agriculture.

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