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Blood mixtures: impact of puncture site on blood parameters

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Abstract Various puncture routes, veins, arteries, heart, are used to take blood in animals. For anatomical reasons, differences in blood composition are expected among puncture sites. However, this issue has been rarely assessed and contrasted results have been reported: strong effects of puncture site versus a lack of effect. We captured free-ranging freshwater turtles from different locations to compare the mean concentrations of 12 blood parameters (metabolites, hormone, ions, and enzyme) among three puncture sites: (1) a lateral branch of the jugular vein, (2) a dorsal subcarapacial cervical plexus (sometimes incorrectly referred as the 'cervical sinus' in the literature), and (3) a caudal plexus site (sometimes incorrectly referred as the 'caudal sinus'). Because we used very small syringes (27–30G), we were able to separate lymph, blood, or blood–lymph mixtures. Our results show very strong effects of puncture site and of mixture level (mean maximal difference between sites was 250 %). We also found strong sex and geographical effects. Typically, there were differences in concentrations of blood solutes sampled from the lateral jugular vein and subcarapacial plexus, mainly due to sampling a mixture of blood and lymph from the 'blood' at the subcarapacial site and pure blood from the lateral jugular site, and likewise, samples from the caudal site were highly variable due to often sampling a mixture of blood and lymph. These results have technical and fundamental implications, especially when performing comparative analyses. Further, by selecting precise puncture sites, physiological differences between lymph and blood compartments could be investigated.

Keywords Corticosterone · Lymph · Plasma metabolites · Hemodilution · Turtle

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Introduction

Blood plays a wide range of roles, delivering water, nutrients, minerals, hormones, and oxygen to cells, transporting effectors of immunity, or carrying wastes to excretory organs for example. Most hematologic parameters vary with time depending on nutritional, stress, disease, and other conditions, and consequently have a diagnostic value. Field researchers have long recognized the benefits of using blood samples to investigate eco-physiological and behavioral issues, or to monitor the health status of individuals (Feder 1987; Feder and Block 1991; Zera and Harshman 2001; Costa and Sinervo 2004).

Each species presents anatomical peculiarities. Therefore, blood is frequently taken from different blood vessels within the body. Investigators usually target the most accessible superficial veins or rely on cardiocentesis (review in Dyer and Cervasio 2008). Although miniaturization permits using very small volumes of blood, and/or continuous monitoring of blood parameters (Seymour 1979; Wess and Reusch 2000; Wiedmeyer et al. 2003; Lefevre et al. 2015), more constraining or lethal methods are sometimes

necessary to collect sufficient amounts of blood (Clarke and Cummins 1982; Hem et al. 1998; Muñoz et al. 2000; Brüssow et al. 2008; Weitten et al. 2013). Overall, various puncture sites are routinely used: caudal or retro orbital blood plexus in laboratory rodents (Van Herck et al. 1998), small ear veins in other mammals (Miller 2003; Michel and Bonnet 2014; Lefevre et al. 2015), flippers in sea mammals (Kurle 2002), the alar vein in birds (Voss et al. 2010), heart in snakes (Fauvel et al. 2012), superficial veins in many taxa (Gottdenker and Jacobson 1995; Fluttert et al. 2000; Crait et al. 2012; Meir and Milsom 2013), and large deep vessels during laboratory experiments (Brüssow et al. 2008; Weitten et al. 2013).

Thousands of field studies based on blood parameters have been published (e.g., Voss et al. 2010 for birds), but a very few assessed the influence of puncture sites (Gottdenker and Jacobson 1995; López-Olvera et al. 2003; Table 1), and none of them considered this factor in comparative analyses (e.g. Promislow 1991). Puncture site might be irrelevant, because, circulation tends to homogenize blood parameters as suggested by several studies (Fluttert et al. 2000; Cuadrado et al. 2003; Miller 2003; Arnold et al. 2008; Hunter et al. 2013). However, other studies, notably clinical experiments, revealed significant differences between puncture sites (Gottdenker and Jacobson 1995; Mylniczenko et al. 2006; Brüssow et al. 2008; Arora 2010), led to ambiguous results (Johnson 1981), or recommended to consider puncture site as an important factor in study design (e.g. Aasland et al. 2010; Mella et al. 2014). Biological causes may explain these discrepancies. Indeed, strong inter-individual variations can mask other effects, especially with small sample sizes. Drastic variations can be caused by sex, health status, reproductive status, captivity, or handling stress for instance (Wells et al. 1984; Bonnet et al. 1994; Aguirre and Balazs 2000; Fazio et al. 2012), but these confounding factors were rarely taken into account (Table 1).

Yet, there are important anatomical reasons to expect hematological differences associated with different puncture sites, notably the widely targeted veins and blood plexuses. It should be noted that in vertebrates, the frequently used term 'blood sinus' (Table 1) is misleading (i.e., a sinus is an open cavity within the body), and it actually refers to plexuses of blood and/or lymphatic vessels (mostly capillaries) that belong to a relatively closed circulatory system. Depending on the puncture site and on the device used, blood puncture may collect a mixture of blood and lymph. Indeed, lymphatic and blood vessels are convoluted and in intimate contact, especially in plexuses. Because capillaries are very small (diameter ranging between 8 and 10 μm), the tip of the needle (e.g., diameter ranging between 0.50 and 0.80 mm for 25–20G needles, the most widely used models) may cut lymphatic and blood vessels during puncture, mixing different liquids in the syringe (i.e., hemodilution). Alternatively, the puncture may target larger vessels without sectioning them, and the liquid retrieved may be pure blood or pure lymph. Because the respective roles, circulation and composition of the blood and of the lymph are different (Walker 1933; Oliver and Detmar 2002; Zawieja 2005), various mixtures should provide contrasted blood compositions. Further, differences in blood chemistry between puncture sites may permit fine examination of organ requirements for resources (e.g., glucose might be less essential for a ligamentous organ like the tail compared to locomotor muscles).

To our knowledge, possible interactions between puncture sites and other important factors (e.g., sex, stress) on blood parameters have not been assessed. Overall, available information regarding methodological and fundamental issues of the effect of puncture site on blood composition is fragmentary. The rapid technical development of portable blood physiology devices prompts the use of blood sampling and hematological assays in an increasing number of species and studies and, thus, creates an urgent need to further explore these issues (Stoot et al. 2014).

In this study, we examined the effect of puncture site on the concentrations of stress hormone (corticosterone), plasma metabolites, and ions (e.g., total proteins, cholesterol, calcium), and one enzyme (alkaline phosphatase) in a freshwater turtle (*Mauremys leprosa*). Chelonians are suitable organisms to explore the impact of puncture sites for several reasons. They are tolerant to manipulation and they exhibit strong inter-individual variations in blood composition (e.g., sex effect; Lagarde et al. 2003). Various puncture sites are used to retrieve blood, including different superficial veins and different plexuses (Jacobson 2000; Table 1). We targeted three contrasted puncture sites: a branch of the jugular vein, a dorsal subcarapacial cervical plexus, and a caudal plexus. We used very small needles (27–30G) to maximize puncture precision, and we used a relatively large number of individuals from different populations including both sexes and different age classes (65 individuals, 129 punctures). Our main goals were: (1) to examine possible effect of puncture site on various blood parameters, (2) to assess possible effect of intrinsic (sex, size) and environmental (disjunct populations) factors, and (3) to contribute to the improvement and standardization of blood sampling techniques.

Materials and methods

Study species

The freshwater turtle *M. leprosa* is a medium size chelonian; females are larger than males (Keller 1997; Bonnet

Table 1 A review of the studies that investigated possible effect of puncture site on blood composition

Taxon	Species	Site	Details	*N*	Status	Blood parameters	Effect	References
Rept	*Gopherus agassizii*	1	JUG	6	M + F	CC, HB, MT, EZ, IO	Y	Gottdenker and Jacobson (1995)
Rept	*Gopherus agassizii*	1	Occipital plexus	6	M + F	CC, HB, MT, EZ, IO		
Rept	*Chelonia mydas*	3[a]	Jug, coccygeal and subcarapacial plexuses	62	M + F	MT, EZ, IO	OV	Hasbún et al. (1998)
Rept	*Testudo marginata*	2	Coccygeal, brachial plexus	7	M + F	CC, HB, MT, EZ, IO	Y/N	López-Olvera et al. (2003)
Rept	*Graptemys geographica*	1	Coccygeal plexus	206	NA	Visual hemodilution	P	Bulté et al. (2006)
Rept	*Graptemys geographica*	1	Subcarapacial plexus	42	NA			
Rept	*Apalone spinifera*	2	Coccygeal, subcarapacial plexus	8	NA	CC, MT, EZ, IO	N/Y	Perpiñán et al. (2010)
Rept	*Caretta caretta*	3[a]	JUG, coccygeal and subcarapacial plexus	101	F	MT, EZ	OV	Fazio et al. (2012)
Rept	*Trachemys scripta*	2	Coccygeal vein, occipital plexus	24	NA	CC, MT	Y	Medeiros et al. (2012)
Rept	*Dermochelys coriacea*	2	Occipital plexus, interdigital vein	12	F	HT, MT, IO, EZ	N/Y	Stewart et al. (2012)
Rept	*Chamaeleo chamaeleon*	2	JUG, tail plexus	11	M + F	CC, MT	N	Cuadrado et al. (2003)
Bird	*Coturnix japonica*	4	JUG, metatarsal and brachial veins, heart	10	RF	CC, HB	Y	Arora (2010)
Bird	*Gallus g. domesticus*	1	Heart	5	RF	CC, HM	N/Y	Johnson (1981)
Bird	*Gallus g. domesticus*	1	Heart	5	RF	CC, HM	N/Y	Johnson (1981)
Bird	*Gallus g. domesticus*	1	Alar vein puncture	5	RF			
Bird	*Gallus g. domesticus*	1	Alar vein cannulation	5	RF			
Bird	*Sterna hirundo*	2	Alar vein, brood patch capillaries	19	NA	HM	N	Arnold et al. (2008)
Bird	*Anas sp. (hybrid)*	2	Alar vein, occipital plexus,	130		HM	Y	Noirault et al. (1999)
Bird	*Struthio camelus*	2	JUG, alar vein	20	M	MT, IO, EZ	N	Moniello et al. (2005)
Mam	*Mus musculus*	2	Orbital plexus, tail plexus	165	F	MT, HM	Y	Rogers et al. (1999)
Mam	*Mus musculus*	3	Orbital plexus, heart, tail plexus	26	F	CC, HB	Y	Nemzek et al. (2001)
Mam	*Mus musculus*	2	Orbital plexus, tail plexus	80	M	MT	Y	Christensen et al. (2009)
Mam	*Mus musculus*	2	Saphenous vein, tail plexus	8	M	MT	Y	Aasland et al. (2010)
Mam	*Mus musculus*	2	Orbital plexus, sub-mandibular vein	20	M	MT, EZ	Y	Fernández et al. (2010)
Mam	*Mus musculus*	2	Heart, tail plexus	90	M	MT	Y	Chan et al. (2012)
Mam	*Mus musculus*	3	Orbital plexus, facila vein, heart	44	F	MT, IN	Y	Mella et al. (2014)
Mam	*Mus musculus*	4	Orbital tail plexus, facial vein, heart, plexus	10–35	M + F	CC	Y	Hoggatt et al. (2015)
Mam	*Rattus norvegicus*	3	Orbital plexus, abdominal aorta, carotid, tail plexus	60	M + F	CC, HB, MT, IO, EZ	Y	Schwabenbauer (1991)
Mam	*Rattus norvegicus*	4	Orbital plexus, vena cava, aorta, tail plexus	12	M	HB	Y	Salemink et al. (1994)
Mam	*Rattus norvegicus*	4	Orbital plexus, dorsal anastomotic orbital vein, abdominal aorta, sublingual vein	80	M + F	CC, MT, IO, EZ	Y	Bernardi et al. (1996)
Mam	*Rattus norvegicus*	2	JUG, tail incision	12	M	HM	N	Fluttert et al. (2000)
Mam	*Rattus norvegicus*	3	Orbital plexus, saphenous and tail veins	5	M	CC, HM, IO, EZ	Y/N	Van Herck et al. (2000)
Mam	*Sus scrofa domesticus*	2	JUG, cava caudalis	19	RF	HM	Y	Virolainen et al. (2005)
Mam	*Sus scrofa domesticus*	2	JUG, cava caudalis	7	RF	HM	Y	Brüssow et al. (2008)

Table 1 continued

Taxon	Species	Site	Details	*N*	Status	Blood parameters	Effect	References
Mam	*Equus caballus*	2	JUG, facial plexus	6	NA	CC, MT	N	Hunter et al. (2013)
Mam	*Equus caballus*	3	JUG, cephalic vein, facial plexus	24	M.F	CC	N	Dahan et al. (2015)
Mam	*Diceros bicornis*	2	Ear vein, radial veins	4	M + F	CC, MT	N	Miller (2003)
Mam	*Bos taurus*	3	JUG, mammary and coccygeal veins	87	NRF	IO	Y	Mashhadi et al. (2009)
Mam	*Canis lupus familiaris*	2	JUG, cephalic vein	23	M + F	CC, MT, IO, EZ	N/Y	Jensen et al. (1994)
Mam	*Homoe sapiens*	3	Peripheral and central veins, radial artery	100		HB, MT, IO	Y/N	Evron et al. (2007)
Amph	*Cyclorana australis*	2	Heart, femoral lymph sac	7	NA	IO, MT	N	Reynolds et al. (2009)
Amph	*Litoria caerulea*	2	Heart, femoral lymph sac	7	NA	IO, MT	N	
Amph	*Bufo marinus*	2	Heart, femoral lymph sac	9	NA	IO, MT	N	
Fish	*Seven shark species*	2	Tail artery, dorsal fin plexus	47	M + F	CC	Y	Mylniczenko et al. (2006)

Site indicates the number of sites punctured per individual

JUG jugular, coccygeal, and subcarapacial "veins" were likely "plexuses" (Fig. 1), brood patch capillaries were sampled using the bug method, *N* sample size, *Status* the sex of sampled animals, *RF* reproductive female, *NRF* non-reproductive females, *NA* not-available, *CC* cell counts and/or hematocrit (e.g., red cells), *HB* hemoglobin (e.g., g/dl) or coagulation, *MT* metabolites (e.g., glucose), *EZ* enzymes (e.g., alkaline phosphatase), *IO* ions (e.g., K^+), *HM* hormones (e.g., progesterone), *Visual hemodilution* hemodilution by lymph was visually estimated, *IN* inflammatory factors, *Effect* significant differences between sites (Y), lack of difference (N), or possible difference (P), *Y/N* difference for most parameters, and conversely for N/Y, *OV* possible site effect was overlooked

[a] Indicates that some information was lacking (e.g., number of individuals sampled per puncture site)

et al. 2010). This turtle inhabits various types of freshwater and brackish waterbodies, and rivers (Keller and Busack 2001). The species is widespread in many regions of Morocco. In April 2013, we captured 66 individuals by hand or using baited traps in different locations (Table 2). The locations are spread along a ~450 km north–south gradient broadly from Fez (34°02′16″N–5°00′12″W), Marrakech (31°37′40″N–7°59′05″W) to Ouarzazate (30°55′12″N–6°52′59″W), thereby encompassing a wide range of habitats and climatic conditions. We first performed the puncture(s) (see below), and then, we recorded sex ($N = 32$ females, $N = 32$ males, $N = 2$ unsexed), body size (straight shell length, SSL), and body mass (BM, ±1 g measured with an electronic scale). SSL and BM were not recorded in one female and in the two unsexed individuals. The mean body size and mean body mass were respectively, 168.6 ± 33.8 mm and 859.8 ± 69.3 g in females ($N = 31$) versus 154.4 ± 41.4 cm and 536.0 ± 68.1 g in males ($N = 32$). Each turtle was permanently marked (shell notches) and rapidly released at the site of capture.

Table 2 Number of male (M) and female (F) *Mauremys leprosa* sampled per study site (location)

Study site	Coordinates	M	F	Und.	Total
Fez (Sebou)	34.0691; 4.9201	5	6	2	13
Moulay Driss (Sebou)	34.0518; 5.5283	6	1	0	7
Meknes (Sebou)	33.8437; 5.5106	1	6	0	7
Marrakech (Tensift)	31.7010; 8.0614	11	9	0	20
Zaouia Ben Sassi (Tensift)	31.6483; 7.8423	2	0	0	2
Ait Ourir (Zat)	31.5599; 7.6770	3	10	0	13
Ouarzazate (lake)	30.9173; 6.7639	4	0	0	4
		32	32	2	66

Fez, Moulay Driss, and Meknes are situated in the Saïs region of Morocco; Zaouia Ben Sassi, Marrakech, Ait Ourir are in the Haouz region; Ouarzazate is a very arid region. The name of the river sampled (Oued) is provided into brackets (artificial lake for Ouarzazate)

Puncture sites and blood collection

We used three different puncture sites (Fig. 1):

1. A small lateral branch of the jugular vein approximately situated at the level of the second–fourth cervical vertebras, often visible protruding beneath the skin. This site is named jugular (vein) for simplicity.
2. A dorsal subcarapacial cervical plexus located in a small hard 'cavity' under the nuchal keratinous scute and above the sixth cervical vertebrae (i.e. at the dorsal basis of the neck). This site contains the dorsal part of the lymphatic ring of the neck (Ottaviani and Tazzi 1977), blood capillaries, and small branches of the jugular vein. This site is usually inappropriately referred as a subcarapacial sinus, cervical sinus, or cervical vein in the literature (note that the anterior basioccipi-

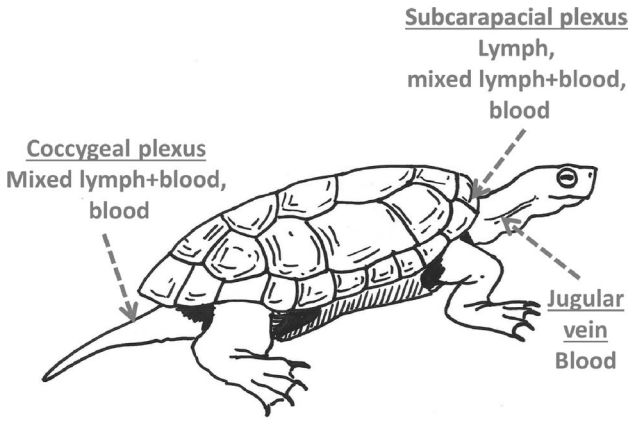


Fig. 1 Three puncture sites were targeted in *Mauremys leprosa*: a lateral branch of the jugular vein, the subcarapacial (cervical) plexus above the sixth cervical vertebrae, and the coccygeal (caudal) plexus near the dorsal base of the tail. Subcarapacial and coccygeal plexuses are often inappropriately referred as 'veins' or 'sinuses' in the literature (Table 1). See text for details about each puncture site (drawing XB)

tal cervical site, approximately above the atlas, was not used). This plexus is not visible and is found by gently pushing the needle in the soft cavity under the nuchal scute. This site is named cervical plexus for simplicity.

3. A caudal or coccygeal plexus that contains a branch of the caudal vein, the caudal lymphatic trunk, plus blood and lymphatic capillaries (i.e., inappropriately referred as caudal sinus or caudal vein). This plexus is not visible and is found by gently inserting the needle along a dorsal medium line in the largest external part of the tail.

Punctures were performed with small needles 27G and 30G connected to a 1 ml syringe. The needle was slightly heparinized (Perpiñán et al. 2010) but the tip of the needle was not passed through the rubber lid of the heparin vial to keep it sharp. Sometimes, the cervical plexus first delivered a crystal clear liquid indicating that lymphatic vessel(s) were targeted (e.g., lymphatic ring of the neck). In these cases, we performed another puncture at the same site, but we slightly pushed the needle forward to obtain a red liquid (presumably blood). In several instances, during cervical and caudal punctures, streaks of red liquid mixed up into a clear liquid, the pink liquid retrieved resulted from mixing lymph and blood. In the jugular vein, we always obtained a dark red liquid. Overall, we obtained six different liquids: (1) jugular blood, (2) cervical plexus blood, (3) cervical plexus mixed blood + lymph, (4) cervical lymph, (5) caudal plexus blood, and (6) caudal plexus mixed blood + lymph. The "plexus blood" category may well contained an unknown (albeit small) amount of lymph.

Table 3 Number of punctures (1–4) performed in male and female *Mauremys leprosa*

Number of punctures	Males	Females	Undetermined	Total
1	31	32	2	65
2	15	19	2	36
3	6	14	0	20
4	4	4	0	8
	57	71	4	129

Three main puncture sites were targeted in random order (Fig. 1). Approximately, half of the tortoises were sampled once ($N = 30$), the others ($N = 36$) were sampled more than once. In several cases, the cervical plexus first delivered lymph exclusively; it was then resampled to collect blood (see text). Consequently, several individuals were sampled four times

Approximately, half of the individuals were sampled only once, whereas others were resampled in one or two additional sites (Table 3). Individuals were randomly assigned to single puncture versus multiple punctures. We randomly changed the order of each puncture site. Because the cervical plexus was sometimes resampled, the total number of punctures per individual was four in several cases. The maximal quantity of liquid taken per puncture was of 1 ml (ranging from 200 µl to 1 ml). We adjusted the quantity of liquid taken to the mass of the turtle (<0.5 % of the body mass).

Immediately after puncture, blood or mixed lymph + blood, were centrifuged at 10,000*g* for 3 min using a field centrifuge; the plasma was then immediately stored in liquid nitrogen (without centrifugation for the lymph). All samples were transported to the laboratory and stored at −25 °C until assays.

Plasma metabolites and enzyme

Plasma metabolite and enzyme levels were measured at the Centre d'Etudes Biologiques de Chizé using an automated chemical analyzer (Pentra C200) supplied by HORIBA Medical Company (©1996–2016 HORIBA, Ltd). All concentrations were determined in accordance with the methodology outlined by the manufacturer. We used commercial assay kits and HORIBA Medical ABX reagents for enzymes or substrates. Three calibrators (ABX Pentra Multical) and a control (ABX Pentra N Control) were systematically introduced in each assay. CVs for intra- and inter-assays were always below 10 % (ranging respectively, between 0.22–8.88 and 1.70–6.34 %).

Total plasma protein and albumin levels were determined by biuret and bromocresol green methods, respectively. Plasma glucose, urea, uric acid, and cholesterol were assayed by enzymatic colorimetric method using commercial kits (HORIBA Medical enzymatic reagents®). Calcium

concentration was measured using o-cresolphthalein complexone (Ca-OCP) method and interference due to Mg^{2+} ions was eliminated by 8-hydroxyquinoline. Plasma triglycerides were determined by the full enzymatic method involving lipase, glycerokinase, glycerol-3 phosphate oxidase, and peroxidase. Alkaline phosphatase (ALP) concentration was measured according to Tietz et al. (1983). Results were expressed in mmol/L for glucose, urea, phosphorus, cholesterol, and triglycerides; in µmol/L for uric acid, albumin, iron, and calcium; in g/L for total proteins; and in U/L for ALP.

Hormonal assays

Corticosterone (CORT) assays were performed using radioimmunoassay in the CEBC laboratory (Bonnet et al. 2013; Dupoué et al. 2014) on a subset of 73 samples (reduced sample size for logistical reasons) of individuals punctured several times ($N = 31$). The steroids were extracted from 40 µl of the sampled tissue (lymph, plasma, and mixed liquid) using diethyl ether (mean extraction rate was of 97.3 ± 5.2 %); the sensitivity of the assay was of 1.9 pg/tube. Cross-reactions with other steroids were low (<0.1 % for 11-deoxy-corticosterone, cortisol, testosterone, and androstenedione, and 7 % for compound S and progesterone). Intra- and inter-assays coefficients of variation remained lower than 4 %.

Handling stress can influence glucocorticoid concentrations, therefore, we measured the amount of time elapsed since capture from the exact time for each puncture minus the exact time at capture (TIME, in min). Although the first puncture was generally rapidly performed after capture (4.1 ± 1.5 min on average, range 2–7 min), this was not systematically the case. Indeed, we voluntarily waited during various amounts of time before the first puncture, and/or between successive punctures to better gauge possible effect of handling stress on CORT. Also, successive samples performed in several individuals required larger amounts of time compared to single puncture.

Analyses

Our main objective was to examine possible effect of puncture site on blood parameters and to assess possible effects (and interactions) of other factors (e.g., sex, size). Therefore, we used generalized linear models with puncture site as the key independent variable and blood parameters as the independent variables. Other factors were also included in the analyses: sex and location (geographic sites) as categorical factors, body mass (BM) and TIME as continuous variables. Although the main focus of this study was puncture site effect, various interactions among discrete and continuous variables were tested. To identify the main independent contributing variables, we used an exhaustive comparison procedure (backward) based on AIC and testing all interactions ($N = 31$ models per blood parameter, total $N = 372$ models).

However, because our sampling design was complex, not fully balanced (due to random resampling procedure, Tables 2, 3), and involved different blood parameters, we adopted a step-by-step and selective approach to limit the inflation of the results. Notably, we did not examine all possible interactions (Rice 1989) since in many instances, the interaction among blood parameters (e.g., between albumin and CORT) could not be easily interpreted because, underlying physiological regulations linking these elements are unknown (if any). To take into account possible pseudo-replicate effect (e.g., several individuals were punctured more than once, Table 3), we retained only the first puncture in the analyses ($N = 65$ individuals), then reperformed the analyses with all samples ($N = 129$; except for CORT due to the smaller initial sample size). In almost all cases, the outcomes were not significantly different using the restricted versus full sample sizes. Therefore, for conciseness, we present the analyses performed on the full data set. In several cases (e.g., glucose), slight differences were observed between analyses ($N = 65$ versus $N = 129$), and both results are then presented. Further, to increase statistical power, we used a restricted number of factors: three puncture sites or three types of liquids (i.e., blood, pure lymph, and pink mixture) instead of six. In this situation, it was possible to assign individual identity as a random factor in the GLM. None of the main outcomes were altered (i.e., regarding the influence of puncture site or liquid type). For conciseness, we did not present these latter results. Instead, we considered that the comparison of the analyses performed with or without pseudo-replicates provided a straightforward mean to gauge the impact of resampling individuals. Finally, we present several results obtained via resampling individuals to provide a direct comparison of the puncture sites on blood parameters (Table 4).

Several variables did not deviate from normal distribution (e.g., albumin concentration, Shapiro–Wilk $W = 0.99$, $p = 0.30$) but others did (e.g., total calcium concentration, Shapiro–Wilk $W = 0.93$, $p < 0.01$). Following log transformation, several variables were normally distributed (e.g., total calcium or glucose concentration, respectively, Shapiro–Wilk $W = 0.98$, $p = 0.07$ and Shapiro–Wilk $W = 0.99$, $p = 0.53$), but not all (e.g. total protein concentration, Shapiro–Wilk $W = 0.92$, $p = 0.01$). However, no dependent variable showed a strongly skewed (except CORT see below) or complex (multimodal) distribution. We visually inspected the distribution of the data (transformed or not) to check for possible outliers. Although, the F test is remarkably robust to deviations from normality and homogeneity of the variances assumptions (see Lindman 1974), we verified that residuals were normally distributed. CORT showed a strongly skewed distribution (many low values);

Table 4 Numbers of samples taken per puncture site among the male and female *Mauremys leprosa*

Puncture site	Males	Females	Undetermined	Total
Jugular-blood	9	11	0	20
Cervical plexus-blood	10	14	2	26
Cervical plexus-mixed	12	12	1	25
Cervical plexus-lymph	9	11	0	20
Tail plexus-blood	15	13	0	28
Tail plexus-mixed	1	8	1	10
	56	71	4	129

Jugular stands for jugular vein; only blood was taken from this puncture site. In the cervical plexus, we obtained dark red liquid (presumably blood), mixed clear and red liquid (mixture of blood and lymph) or only crystal clear lymph. In the tail, we obtained either dark red liquid or pink mixed blood and lymph

Table 5 Results from generalized linear models analyses; only the significant effects retained through model selection based on AIC are presented (see text for details)

Parameter	Effect	*Df*	Wald Stat	*P*
CORT	Intercept	1	12.79	0.001
	TIME	1	14.81	0.001
	BM	1	7.023	0.008
	Puncture site	5	12.66	0.027
	Location	6	14.02	0.029
Albumin	Intercept	1	3055.46	0.001
	Puncture site	5	38.90	0.001
	Location	6	13.24	0.040
Total proteins	Intercept	1	433.23	0.001
	Puncture site	5	50.73	0.001
Glucose	Intercept	1	129.80	0.001
	TIME	1	50.61	0.001
	Puncture site	5	19.89	0.078
	Location	6	36.42	0.001
Glucose[a]	Intercept	1	99.77	0.001
	TIME	1	27.42	0.001
	Puncture site	5	13.46	0.019
	Location	6	23.14	0.001
Cholesterol	Intercept	1	24.97	0.001
	Puncture site	5	26.88	0.001
Triglycerides	Intercept	1	39.43	0.001
	Sex	1	4.93	0.026
	Puncture site	5	12.27	0.031
Uric acid	Intercept	1	386.72	0.001
	TIME	1	9.18	0.002
	BM	1	25.40	0.001
	Puncture site	5	21.62	0.001
	Location	6	87.03	0.001
Uric acid[a]	Intercept	1	227.04	0.001
	TIME	1	2.42	0.120
	BM	1	13.21	0.001
	Puncture site	5	9.48	0.091
	Location	6	48.16	0.001
Urea	Intercept	1	2.73	0.093
	BM	1	16.10	0.001
	Location	5	31.16	0.001
Urea[a]	Intercept	1	0.34	0.562
	BM	1	3.88	0.049
	Location	5	6.54	0.369
Calcium	Intercept	1	59.53	0.001
	BM	1	5.70	0.020
	Puncture site	5	23.58	0.001
Phosphorous	Intercept	1	25.73	0.001
	Sex	1	8.54	0.003
	Puncture site	5	130.78	0.001
Iron	Intercept	1	0.84	0.359
	Sex	1	3.88	0.049
Alkaline phosphatase	Intercept	1	51.11	0.001
	Puncture site	5	28.31	0.001
	Location	6	54.66	0.001
Alkaline phosphatase[a]	Intercept	1	23.68	0.001
	Puncture site	5	23.65	0.001
	Location	6	9.96	0.126

The results presented are based on the full sample size (including several cases where more than one sample was collected per individual), except when differences were observed with analyses performed on a restricted sample (pseudo-replicates discarded, indicated with[a])

therefore, we used a Poisson distribution in the analyses. For several samples, the amount of liquid collected was insufficient to perform all assays, resulting in slight variations of sample size per blood parameter assayed (*N* ranging from 110 to 129). Analyses were performed with STATISTICA 12.0 (StatSoft 2013, http://www.statsoft.fr).

Ethical note

We never observed any sign of distress in any individual (using very small needles prevented hemorrhagic problems that are regularly observed with large needles). All procedures were performed in accordance with the international regulations. Permits for population monitoring # 234/12 HCEFLCD/DLCDPN/DPRN/CFF and 05/2013 HCEFLCD/DLCDPN/DPRN/CFF were issued by the High Commissariat for Water and Forest (Morocco).

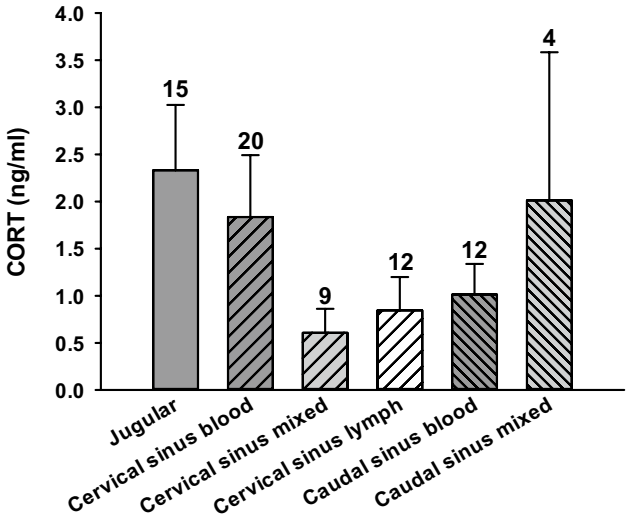


Fig. 2 CORT varies with puncture sites (Table 5). Mean values were corrected by TIME (handling time), presented with SD and sample size. Sexes and locations were pooled. Note the small sample size of the mixed blood/lymph caudal plexus. See text and Fig. 1 for details about puncture sites

Ethical procedures were approved by the ethical committee COMETHEA (permit # CE2013-6).

Results

Effect of handling stress

The amount of time elapsed since capture (TIME) ranged from 2 to 251 min (52.5 ± 59.5 min on average, $N = 130$), and positively correlated with several blood parameters, CORT ($F = 19.88$, $r = 0.47$, $P < 0.001$, $N = 73$), and glycemia ($F = 65.54$, $r = 0.58$, $P < 0.001$, $N = 129$) notably. Therefore, this factor was also implemented in the analyses.

CORT

Selection based on AIC suggested that the best model included TIME, BM, puncture site, and location (Table 5). Higher CORT levels were observed in the jugular blood and the lowest in the lymph or mixed blood/lymph from the cervical plexus (Fig. 2).

Plasma metabolites and ions

The explanatory variable(s) retained through model selection differed among the plasma metabolites examined (Table 5). Concentrations were strongly, and sometimes exclusively, influenced by puncture site (albumin, total proteins, glucose, cholesterol, triglycerides, uric acid, calcium, and phosphorous); except for urea and iron. Other factors were also important, notably location and TIME, and to lesser extent sex and BM, with marked differences among blood parameters.

Higher plasma metabolite and iron concentrations were generally observed in the blood (e.g., jugular) compared to hemodiluted liquids, and the lowest values were generally recorded in the lymph (Fig. 3a, b). However, we observed several important deviations from this general pattern (Fig. 3a, b).

Alkaline phosphatase

Enzyme concentrations were influenced by puncture site and location (Table 5), with higher values observed in the blood compared to hemodiluted mixture and lymph (Fig. 3b).

Direct comparison of resampled individuals

In several instances, individuals were sampled at two different sites (Table 3). We focused on jugular blood (not hemodiluted) versus cervical sinus blood (a widely used site). These two sites were sampled in a random order. Despite a reduced sample size (due to the random selection among three puncture sites and the distinction among six liquids), significant differences were found. For instance, CORT was higher in the jugular blood compared to the cervical blood (Wilcoxon test for paired samples, $Z = 2.65$, $P < 0.01$, $N = 9$), and similarly, total protein concentrations were higher in the jugular blood ($Z = 2.41$, $P = 0.02$, $N = 11$; Fig. 4).

Discussion

The three main puncture sites we targeted (one vein and two plexuses; six sub-sites considering the differences between blood and lymphatic vessels) are routinely used for blood sampling chelonians (Table 1). Different sites represented by superficial veins and plexuses are also routinely used in other vertebrates (Table 1). Therefore, our results regarding the impact of three main puncture sites on a wide range of blood parameters provide new insights into vertebrates and, thus, are of general interest for field, ecological, and clinical investigations. Previous studies compared only two sites, overlooked site effects, or relied on invasive/laboratory methods not easily applicable in the field (Table 1).

Puncture site influences all blood parameters tested

Our results clearly demonstrate that puncture site exerts a significant influence on plasma concentrations of all the

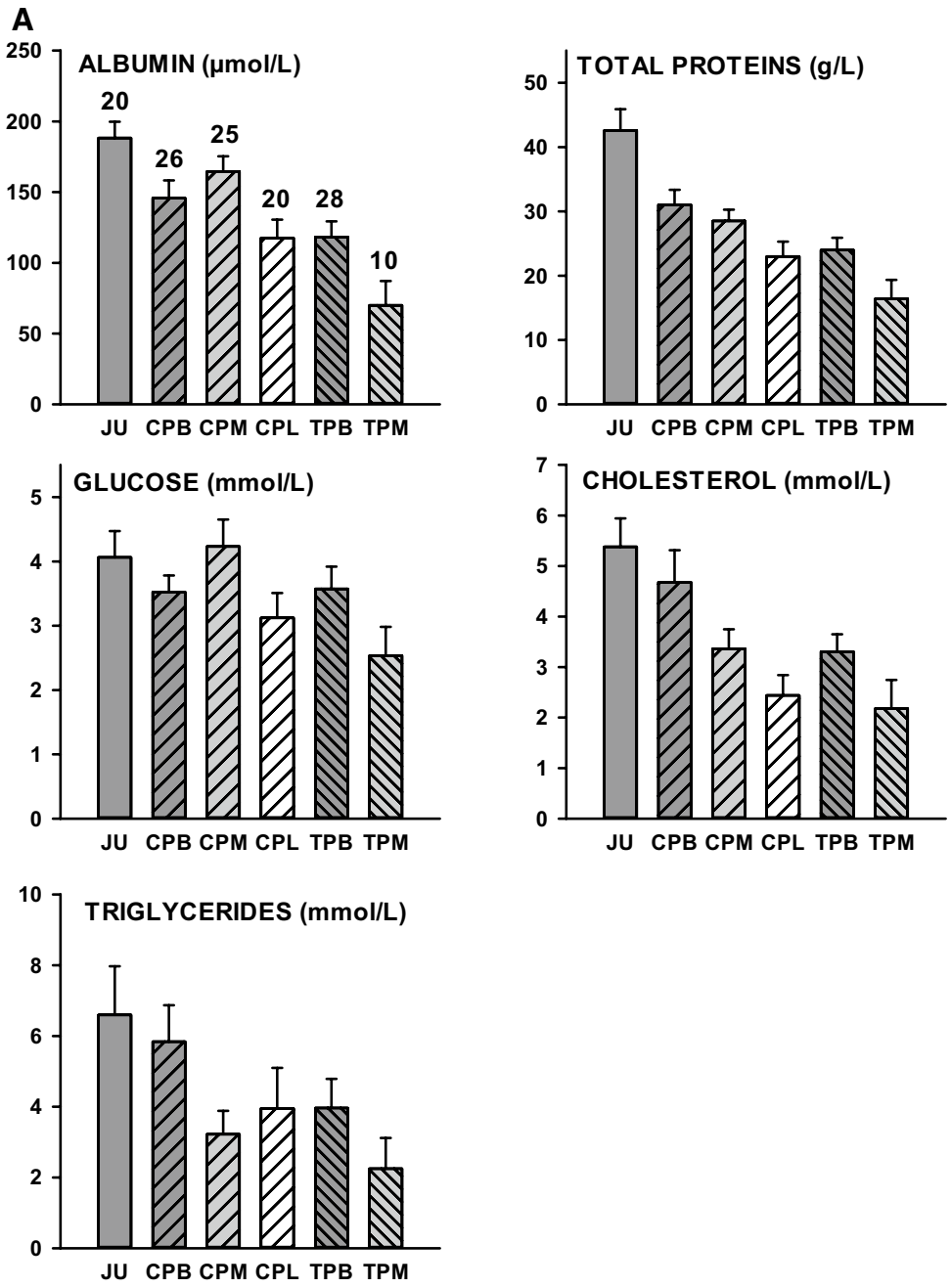
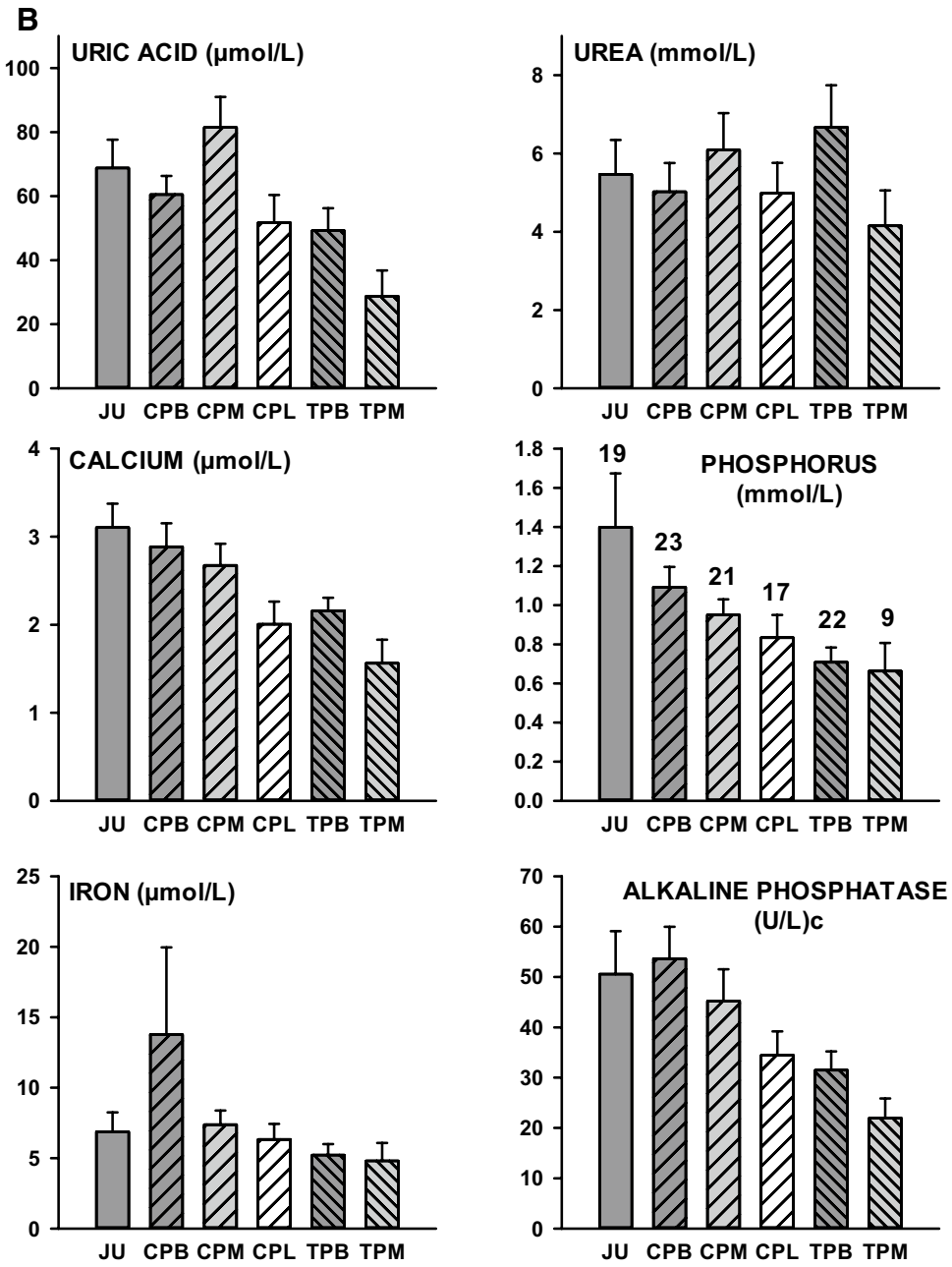


Fig. 3 **a**, **b** Mean values of different blood parameters in function of puncture site. Means are provided with SE. Sample sizes indicated for albumin are similar for all parameters, except phosphorus. *JU* jugular, *CPB* cervical plexus blood, *CPM* cervical plexus mixed (blood + lymph), *CPL* cervical plexus lymph, *TPB* tail plexus blood, *TPM* tail plexus mixed

metabolites, ions, hormones, and enzymes assayed. The maximal difference between the mean values recorded in different puncture sites was substantial, averaging 250 ± 6 % (±SD, $N = 12$ parameters measured, range 160–380 %, respectively, for urea and CORT). We found strong differences between jugular vein and the sampled plexuses, but also between and within cervical and caudal plexuses (Fig. 3a, b). Consequently, substantial imprecision can emerge when puncturing different sites or when puncturing a single site, especially when large needles are used.

The parameters assayed in this study do not encompass the whole range of elements that circulate in the blood. They, nonetheless, represent a wide spectrum: To the best of our knowledge, the current study is the first that measured hormone, enzyme, ion, and plasma metabolite concentrations. Moreover, although we did not perform erythrocyte counts, hematocrit or hemoglobin quantification, there is little doubt that the crystal clear lymph extracted from plexuses would have exhibited significantly lower values compared to the dark blood extracted from the jugular. Similarly, lymphatic vessels carry specific and variable

Fig. 3 continued



amounts of leukocytes compared to veins, and major differences with blood vessels are expected (Oliver and Detmar 2002). Further investigations are necessary to examine to what extent our results are applicable to other blood parameters and other taxa, and to better assess the discrepancies between previous studies.

Technical issues

Although small sample sizes pose statistical difficulties, they characterize most ecological studies performed in free-ranging individuals, and several clinical laboratory experiments (Table 1). In many studies, the puncture site is not indicated. Further strong variations of blood parameters can be induced by environmental and intrinsic factors. For example, handling can rapidly trigger strong stress responses with an increase of several hormone and metabolite concentrations (Arnold et al. 2008; this study) or a decrease in others (Michel and Bonnet 2014). Substantial variations also exist among individuals, sexes or populations, notably in ectothermic species. Depending upon the parameter tested, our results revealed all these effects (Table 5). A study in a terrestrial tortoise also showed marked effects of sex, seasons, and years (climate)

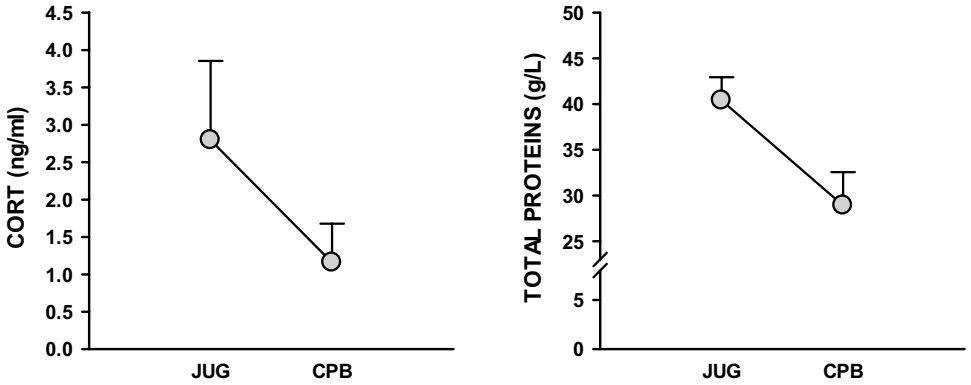


Fig. 4 Direct comparisons of concentrations of two key solutes sampled at two different sites for the same individuals. Lateral jugular vein (JUG) that provides pure blood and the cervical plexus (a widely used site). We retained only the supposedly not hemodiluted blood samples from this second site (CPB, see text for details). Therefore, we attempted to compare 'pure blood' retrieved from two sites. Sample sizes are 9 individuals resampled for CORT and 11 for total proteins

on various blood parameters (Christopher et al. 1999), but these factors have been overlooked in puncture site studies (Table 1). Multiple (random/alternating) sampling on the same individual provides a robust mean of investigation, and it almost systematically showed marked site effects (Table 5; Fig. 4). Previous results suggesting a lack of puncture site effect based on small sample sizes or on indirect comparisons should be examined with caution (e.g., similar incongruences among hormonal studies have been clarified with large samples; Bonnet et al. 2001).

The device and method employed to take blood are important; significant effects of the anticoagulant agent have been found, but no effect of needle size was reported (Miller 2003; Perpiñán et al. 2010). In the current study, we used smaller needles (27–30G) than previously used, enabling us to target blood or lymphatic vessels with a relatively higher accuracy. Yet, we obtained unmixed lymph only in the cervical plexus. The impact of needle size is likely important when puncturing plexuses, but less important when puncturing large blood vessels (e.g., jugular, ear veins), and this impact may vary with the studied species (e.g., rhinoceros versus mouse). Unfortunately, needle size is rarely reported in scientific publications. Investigations focusing specifically on this issue are needed, notably because, relatively large needles (20–23G, which may cut vessels and tissues thereby mixing liquids) are the most commonly used, even on small species. Moreover, hemodiluted samples were sometimes arbitrarily discarded by researchers (upon visual inspection), precluding investigations about possible effect of puncture site (e.g. Hasbún et al. 1998; Fazio et al. 2012). Visual inspection was rarely taken into account through a crude visual scoring (Drake et al. 2012). Overall, although blood sampling techniques have been reviewed (Dyer and Cervasio 2008), the impact of puncture site and lymph hemodilution on blood parameter was not fully assessed, particularly in natural populations, and several technical issues have been insufficiently scrutinized.

Environmental and intrinsic factors

Discussing all the significant effects we found (Table 5) is out of scope of the current study. However, several results highlight the possible importance of sampling site to accurately assess biological and environmental factors. A significant sex effect was observed for several parameters (Table 5). For instance, plasma concentrations of triglycerides were higher in females (5.73 ± 5.54 mmol/L) compared to males (2.53 ± 2.28 mmol/L). This sex difference was expected, because, we sampled animals during vitellogenesis, a process that markedly influences blood composition (Bonnet et al. 1994; Christopher et al. 1999; Lagarde et al. 2003). However, further analyses suggest that this sex effect was significant only in blood taken in the jugular or in the cervical plexus (Fisher LSD post hoc tests $P < 0.001$), whereas non-significant effects were found using the blood from the caudal plexus, lymph, and mixtures (Fisher LSD post hoc tests $0.06 < P < 0.99$). Thus, analyses based on blood samples taken from different puncture sites provide different results and lead to different conclusions: significant versus lack of effects.

Similar complications were found in other blood parameters. Considering the wide north–south geographical gradient where populations where sampled, hematological differences were expected. Indeed, our populations were exposed to contrasted environmental conditions and belonged to different subspecies (Fritz et al. 2006). Differences among locations (e.g., albumin levels) were significant when comparing jugular samples, but not caudal plexus samples. As above, jugular and cervical plexus

bloods provided more discriminant results compared to the caudal plexus blood. This differential discriminatory capacity of the analyses among puncture sites was not due to sample sizes; the blood extracted from the tail ($N = 28$) offered more statistical power than the jugular blood ($N = 20$).

Our results suggest that jugular or cervical blood should be preferred over caudal sampling to perform precise eco-physiological investigations. A similar recommendation was already proposed by Gottdenker and Jacobson (1995) and Christopher et al. (1999). Yet, other puncture routes might be preferable for practical reasons and for other questions; the cervical plexus is particularly easily sampled in various chelonians.

Conclusions

Our results agree with previous clinical studies that reported very strong effects of puncture site, even when small sample sizes were used (Gottdenker and Jacobson 1995). Because we used a relatively large sample, assayed various parameters, employed small needles, did not arbitrarily discard hemodiluted samples, and considered both intrinsic and environmental factors, our analyses combined technical precision with statistical robustness. For physiological and anatomical reasons, major differences were expected among sampling sites. Previous investigations comparing various blood parameters using sufficient samples in standard conditions always found strong puncture site effects (Table 1), except when two different superficial well-identified veins were compared for instance (Moniello et al. 2005). We hypothesize that previous negative results suffer from methodological weaknesses (e.g., small sample size combined with strong inter-individual heterogeneity). On average, our study and previous reports show that concentrations of most parameters were higher in the jugular blood compared to the lymph or to the mixtures between blood and lymph retrieved from plexuses. However, we did not find any unequivocal pattern and each parameter assayed displayed a specific profile.

In addition to these methodological cautions, physiological issues should be considered. For example, using small needles, lymph samples can be collected and analyzed. Experimental calculations indicate that the volume of lymph is roughly equal to the volume of blood in mammals (Warren 1940; Tretbar 2008); thus, lymph represents a major tissue. Important regulations are expected, in terms of hydro-mineral balance and adaptation to climatic conditions for example. By carefully targeting different puncture sites, physiological regulations and, thus, trade-offs could be investigated. Thus, the complications associated with puncture site effects also open research opportunities.

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Compliance with ethical standards

Conflict of interest The authors declare that no competing interest.

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Data availability The dataset used in this study would be deposited in the dryad platform (http://datadryad.org).

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