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Hematology and Plasma Biochemistry in Whale Sharks (*Rhincodon typus*): Baseline Reference Intervals Based on Captivity Status, Blood Sampling Sites, and Handling Methods

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This study aimed to establish reference intervals for red and white blood cell counts, hematocrit levels, mean corpuscular volume, and 25 key plasma biochemistry parameters in captive and wild whale sharks (*Rhincodon typus*). Blood samples were collected from the caudal vein (CV) and dorsal cutaneous vein (DCV) of 30 wild sharks caught in fixed nets off the Kochi Prefecture coast, Japan, and from 24 captive sharks between 2007–2023. Samples were obtained from restrained captive and wild sharks as well as unrestrained captive sharks trained for husbandry. Comparative analyses considered three factors: captivity status (wild vs. captive sharks under restraint), blood sampling sites (CV vs. DCV under restraint), and handling methods (DCV sampling under restrained vs. unrestrained conditions). Analysis of captivity status revealed significant differences in 12 of 29 parameters, with triglyceride levels significantly lower in wild sharks, possibly indicating nutritional deficiencies due to their prolonged migrations. Comparisons of blood sampling sites revealed significant differences in 11 parameters, including red and white blood cell counts and hematocrit levels, with most CV-derived parameters being higher than those from the DCV. A strong correlation ($r > 0.7$) was found between the CV and DCV for 19 parameters, indicating predictive values between these vessels. Additionally, the relationship between RBC, Ht,

and MCV indicates that the RBC and MCV results may not be entirely reliable and should therefore be interpreted with caution. In the handling method comparison, eight parameters exhibited significant differences; specifically, aspartate aminotransferase, ammonia, and creatine phosphokinase levels were likely influenced by stress effects, including restraint-induced muscle damage. These findings emphasize the importance of unrestrained blood collection, facilitated through husbandry training, for accurate blood parameter evaluations. Integrating statistical results across the three studied factors allowed for the establishment of reference intervals, means, and medians for whale sharks, contributing to health management in captive sharks and conservation in wild populations.

Key words: Shark health assessment, Comparative blood analysis, Captive vs. wild comparison, Husbandry training

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BACKGROUND

As the largest fish species, the whale shark (*Rhincodon typus*) reaches lengths of up to approximately 19 m (McClain et al. 2015). It is widely distributed across tropical and warm subtropical regions (Colman 1997; Rowat and Brooks 2012; Sequeira et al. 2014), exhibiting extensive migratory behavior, with specific regions identified as high-density aggregation “hot spots” (Sequeira et al. 2013). The relationship between whale sharks and humans includes not only ecotourism at these hot spots but also research on shark physiology and behavior in aquariums (Black et al. 2013; Hara et al. 2018; Matsumoto et al. 2019) along with environmental education for visitors. However, whale shark is currently listed as “Vulnerable” on the International Union for Conservation of Nature Red List due to anthropogenic impacts, including targeted fishing, bycatch, and vessel collisions (Pierce and Norman 2016).

Since 1990, Osaka Aquarium Kaiyukan has engaged in the care and management of whale sharks for research and education. To minimize impacts on wild populations, whale sharks are temporarily kept at the aquarium and released during migration seasons near Japanese waters. To track their migration routes, pop-up satellite archival tags are affixed to the sharks. When held in captivity, the sharks are behaviorally observed and physiologically assessed to refine management

practices, with practices including blood testing on free-swimming sharks using husbandry training techniques (Sodeyama et al. 2012).

In aquarium-based marine mammals, blood parameter evaluation is widely used for health assessments (Bossart et al. 2001). However, data on elasmobranch blood parameters remain limited. For specific species, studies have reported baseline blood parameters in both wild (e.g., AtallahBenson et al. 2020; Starostinetsky-Malonek et al. 2023) and captive populations (e.g., Morón-Elorza et al. 2022; Hyatt et al. 2016), as well as blood parameter comparisons between these populations (e.g., Grant and Campbell 2020; Cusack et al. 2016). In addition, changes in blood parameters due to capture stress (e.g., Falco et al. 2023), fasting, and illness (e.g., Wosnick et al. 2020), reproductive stages (e.g., Nau et al. 2018), and sampling sites (e.g., Phillips et al. 2016) have been explored. Nevertheless, the reference values and mechanisms underlying blood parameter variations in many wild and captive elasmobranchs remain unclear. In the three published studies on whale shark blood parameters (Dove et al. 2010 2022; Ueda et al. 2017), descriptive statistics were reported without reference intervals (RIs) based on inferential statistics. Therefore, establishing RIs is crucial for detecting health issues in sharks, improving husbandry practices in captive populations, and assessing the effects of environmental changes on wild populations (Arnold and Delaune 2022).

From spring to autumn, whale sharks migrate along the Kochi Prefecture coast, Japan (Matsunaga et al. 2003). During 2007–2023, blood samples from 30 wild sharks captured in fixed nets along this coast and 24 sharks at Osaka Aquarium Kaiyukan were collected from the caudal vein (CV) and dorsal cutaneous vein (DCV), which correspond to the primary vascular system (PVS) and the secondary vascular system (SVS), respectively. Manual blood cell counts and biochemical values were measured using a point-of-care blood chemistry analyzer. Using these blood data, the present study aimed to enhance whale shark welfare through medical management of captive sharks while contributing to wild population conservation by establishing and comparing RIs based on three factors: (1) captivity status (wild vs. captive sharks under restraint), (2) blood sampling sites (CV vs. DCV sampling under restraint), and (3) handling methods (DCV sampling under restrained vs. unrestrained conditions).

MATERIALS AND METHODS

Wild Whale Shark Capture, Transport, and Blood Sampling

Wild whale shark data were collected from 30 sharks (total length: 310–694 cm; 22 males, 8 females) captured in fixed nets at 12 locations along the Kochi Prefecture coast during 2007–2023. Blood samples were taken from the CV and/or DCV the day after capture, with the sharks restrained near the water surface in tightened nets (*i.e.*, restrained; Fig. 1). Following blood collection, sharks were evaluated for sex, size, body proportions, and external injuries. Sharks deemed unsuitable for captivity were released back into the wild, whereas those considered suitable were transferred to Osaka Aquarium Biological Research Institute of Iburi Center (OBIC) in Kochi Prefecture. Whale sharks captured near OBIC were transported by a towing vessel, whereas those captured farther away were transported to a nearby fishing port using the towing vessel, placed in a container designed for whale sharks, and transported by truck to OBIC. Blood samples were also collected just before sharks were released into OBIC tanks, and the blood data were included in the wild shark dataset for analysis.

Fig. 1. Blood sampling from the caudal and dorsal cutaneous veins of a restrained whale shark (*Rhincodon typus*). An underwater blood collection kit was used for sampling.

All blood samples were collected and processed following standardized procedures. Equipment included a 10-mL syringe with an 18-gauge needle for CV sampling and a syringe with a 21-gauge needle for DCV sampling. Insertion points in the CV and DCV were the ventral side near the base of the tail fin and the tail side near the base of the second dorsal fin, respectively. Given the underwater sampling conditions, a setup similar to that described by Ueda et al. (2017) was employed. Heparin was used as an anticoagulant, and typically 5–10 mL of whole blood was

collected. No controlled feeding occurred between capture and sampling, although incidental consumption of natural prey cannot be excluded.

Captive Whale Shark Transport, Management, and Blood Sampling

Captive whale shark data were obtained from 24 individuals (total length: 310–630 cm; 16 males, 8 females; captivity duration: 114–3,299 days) initially captured in fixed nets at eight locations along the Kochi Prefecture coast, assessed as suitable for captivity, and placed under care in two OBIC tanks and the Pacific Ocean Tank at Osaka Aquarium Kaiyukan.

The two OBIC tanks were each 5-m deep, with capacities of 3,000 m^3 and 1,000 m^3, respectively, and neither tank was publicly accessible. Both tanks had semiclosed recirculation systems using well seawater with 32%–35% salinity. Conditions differed slightly: the 1,000-m^3 tank maintained a temperature of 22.3°C–25.5°C and pH of 7.67–8.16, whereas the 3,000-m^3 tank had a temperature of 23.4°C–26.9°C and pH of 7.44–8.03. Both tanks maintained ammonia (NH_3) and nitrite levels below 0.20 and 0.05 ppm, respectively. Whale sharks were housed either alone or with small fish in the 1,000-m^3 tank and with another whale shark and small fish in the 3,000-m^3 tank.

After acclimatizing at OBIC for a sufficient period, whale sharks suitable for display were transported by truck to Osaka Aquarium Kaiyukan and housed in the 9-m-deep, 5,400-m^3 Pacific Ocean Tank, which was publicly accessible. This tank, equipped with a similar semiclosed recirculation system, used seawater from the Kuroshio current off Wakayama Prefecture. It maintained a water temperature of 20.1°C–25.6°C, pH of 7.37–7.83, salinity of 34%–37%, and NH_3 and nitrite levels below 0.20 and 0.05 ppm, respectively. Another whale shark, along with various teleosts and elasmobranchs, was housed in this tank.

Captive whale sharks were fed twice daily (morning and afternoon), primarily with krill and mysid shrimp, supplemented with sakura shrimp, whitebait, artificial shark feed, and vitamins. The feed, a mix of 2–4 types, was adjusted based on shark size and health, provided at 0.4%–0.9% of their body weight per day.

The equipment and methods for blood sampling in captive whale sharks were the same as those used for wild sharks. Sampling was conducted when transferring sharks between OBIC tanks or between OBIC tanks and the Pacific Ocean Tank at Osaka Aquarium Kaiyukan. Transfers occurred around 15–21 h after feeding. Sharks were placed in transport containers, where they were unable to swim (i.e., restrained), and blood was drawn from the CV and/or DCV. For sharks trained using desensitization techniques, including diver approaches, body surface contact, and needle stimulation near the base of the second dorsal fin, blood sampling from the DCV was performed without restraint (*i.e.*, unrestrained) while the sharks swam during feeding (Fig. 2).

Fig. 2. Blood sampling from the dorsal cutaneous vein of an unrestrained whale shark (*Rhincodon typus*). Husbandry training was applied to facilitate sampling.

Table 1 provides all relevant information pertaining to wild and captive whale sharks. All animal procedures were approved by Kaiyukan and Nifrel Research Ethics Review Committee (permit number: KN24001).

Table 1. Whale shark (*Rhincodon typus*) data from sampling conducted during 2007–2023

Sex	Total length (cm)	Duration of captive	Sampling number				
			Wild (CV, Restrained)	Wild (DCV, Restrained)	Captive (CV, Restrained)	Captive (DCV, Restrained)	Captive (DCV, Unrestrained)
M	410–550	1503	1	1	1	1	0
M	435–522	1392	1	1	2	4	0
M	440	183	1	2	1	1	0
M	440	1068	2	2	0	1	0
M	432–475	855	2	2	8	8	29
M	460–540	1154	0	1	1	1	36
M	380–560	1588	0	2	3	4	50
M	480	424	4	0	1	0	0
M	390	159	2	2	1	1	3
M	380–555	1480	2	2	1	4	17
M	350–450	867	2	2	3	3	23
M	428–525	1421	1	1	2	2	43

M	466–499	483	0	3	1	1	19
M	440–500	382	0	3	0	0	12
M	310–410	407	0	3	1	2	14
F	370–630	2147	0	1	0	1	1
F	476–523	614	2	2	1	1	19
F	450	114	2	2	2	2	0
F	410–506	1075	2	2	2	2	27
F	410	234	1	2	1	1	1
M	400–496	2262	0	0	1	2	0
F	410–525	1121	0	0	9	8	25
F	360–460	699	0	0	2	4	13
F	487–600	3299	0	0	3	3	104
M	694		1	1	0	0	0
M	450		0	1	0	0	0
M	420		1	1	0	0	0
M	650		1	1	0	0	0
M	450		1	1	0	0	0
M	450		0	1	0	0	0
M	530		0	1	0	0	0
F	550		0	1	0	0	0
F	470		0	1	0	0	0
F	450		0	1	0	0	0

M and F indicate male and female, respectively. Regarding Sampling number, Wild (CV, Restrained), Wild (DCV, Restrained), Captive (CV, Restrained), Captive (DCV, Restrained), Captive (DCV, Unrestrained) indicate blood samples from the CV of wild restrained sharks, DCV of wild restrained sharks, CV of captive restrained sharks, DCV of captive restrained sharks, and DCV of captive unrestrained sharks, respectively.

Blood Analyses

For sharks at Osaka Aquarium Kaiyukan and OBIC, blood was centrifuged immediately after collection to separate plasma. For wild sharks, blood samples were initially refrigerated, with part of the sample centrifuged at OBIC within a few hours and the resulting plasma frozen. All samples were then transported to Osaka Aquarium Kaiyukan for analysis.

Red blood cell (RBC) and white blood cell (WBC) counts were measured following the method of Walsh and Luer (2004). Approximately 40 μL of blood was transferred to hematocrit (Ht) tubes for centrifugation at 11,000 rpm and room temperature for 5 min using a Hematocrit Centrifuge MC-202 (Hitachi Koki Co., Ltd., Tokyo) to measure Ht levels. Mean corpuscular volume (MCV) was calculated based on RBC counts and Ht levels. The remaining blood was centrifuged at 3,000 rpm for 5 min using an AcNo-3 centrifuge (Sagami Corporation, Kanagawa, Japan), and the obtained serum was analyzed using a Fuji DRI-CHEM 7000V analyzer (Fuji Medical Systems, Co. Ltd., Tokyo) to assess the following biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ-glutamyltransferase (GGT), total bilirubin (T-Bil), total protein (TP), albumin (Alb), NH_3, creatine phosphokinase (CPK), triglyceride (TG), total cholesterol (T-Cho), blood urea nitrogen (BUN), uric acid (UA), creatinine (Cre), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphate (iP), magnesium (Mg), glucose (Glu), amylase (Amy), and lipase (Lip). Plasma osmolality (PO) was measured in a clinical laboratory using freezing point depression.

Statistical Analyses

Aside from establishing RIs, statistical analyses were conducted using EZR version 1.65 (R Commander version 2.9-1) (Kanda 2013). All tests used a significance level of $p < 0.05$. Initial health assessments were based on physical injuries, body condition, in addition to behavioral and feeding observations for captive sharks. Blood samples from sharks not meeting health standards were excluded from the dataset. However, health evaluations were challenging due to limited clinical data and handling difficulties associated with large sharks. In wild sharks, health assessments shortly after capture in fixed nets were also constrained, meaning that some blood samples may have come from individuals deemed unhealthy. Establishing Ris for wild animals often involves limited specimen availability, few established health markers, and difficulty assessing health in large or unfamiliar species, which can lead to uncertain evaluations or outlier inclusion. To ensure that data reflects only healthy individuals, outlier tests are recommended to identify and exclude unhealthy individuals and abnormal data points (Friedrichs et al. 2012). In the present study, blood data were grouped based on combinations of the three studied factors: captivity status, blood sampling site, and handling method. Outliers in each group were identified and excluded using the Smirnov–Grubbs test. The groups were as follows:

Captive (CV, Restrained): Blood samples from the CV of captive, restrained sharks.
Captive (DCV, Restrained): Blood samples from the DCV of captive, restrained sharks.
Captive (DCV, Unrestrained): Blood samples from the DCV of captive, unrestrained sharks.
Wild (CV, Restrained): Blood samples from the CV of wild, restrained sharks.
Wild (DCV, Restrained): Blood samples from the DCV of wild, restrained sharks.

For analysis of captivity status and blood sampling site data, Captive (DCV, Unrestrained) samples were excluded. Logarithmic transformation, with or without adding 1, was implemented before performing two-way ANOVA if needed. Based on Kolmogorov–Smirnov test outcomes, either the Pearson product-moment correlation coefficient or Spearman's rank correlation coefficient was used for correlations. Extremely low *p*-values were reported as $p < 0.001$. For handling method analysis, only two groups, Captive (DCV, Restrained) and Captive (DCV, Unrestrained), were considered, with logarithmic transformation applied before conducting Student's *t*-test as necessary.

Based on the results of statistical tests for each factor (captivity status, blood sampling site, and handling method), blood parameters without significant differences within each factor were combined. Descriptive statistics (mean, median, standard deviation, minimum, and maximum) and

Ris were calculated using the combined data. Ris were determined for parameters with a sample size of ≥ 20, following American Society for Veterinary Clinical Pathology guidelines (Friedrichs et al. 2012), using Microsoft Excel 2019 and Reference Value Advisor V 2.1 (Geffré et al. 2011). For parameters exhibiting non-Gaussian distributions, a robust method was applied following Box–Cox transformation. For Gaussian distributions, the standard method was employed, with or without Box–Cox transformation, depending on data distribution. Some parameters had limited dataset sizes and skewed distributions, leading to low Ris and confidence intervals that could not be ascertained by the software, yielding negative values. These values were replaced with zero. Additionally, Ris could not be established for parameters where Box–Cox transformed data failed to conform to a Gaussian distribution.

RESULTS

Comparison of Captivity Status and Blood Sampling Sites

After excluding outliers, blood samples in the Captive (CV, Restrained), Captive (DCV, Restrained), Captive (DCV, Unrestrained), Wild (CV, Restrained), and Wild (DCV, Restrained) groups included 45 samples from 21 sharks, 51 samples from 20 sharks, 436 samples from 19 sharks, 29 samples from 20 sharks, and 46 samples from 29 sharks, respectively. Mean values for each parameter from each shark were calculated from these data and used for statistical testing. However, since not all blood samples were tested for every blood parameter, it was not possible to calculate mean values for certain parameters. As a result, the number of mean data values for each blood parameter was 13–21, 12–20, 14–18, 9–20, and 14–29 in the Captive (CV, Restrained), Captive (DCV, Restrained), Captive (DCV, Unrestrained), Wild (CV, Restrained), and Wild (DCV, Restrained) groups, respectively.

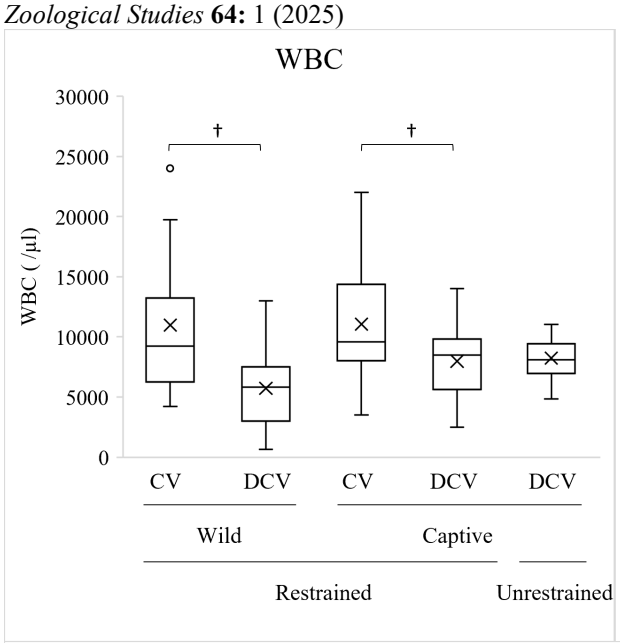
Table 2 presents descriptive statistics and RIs based on two-way ANOVA of blood parameters, considering captivity status and blood sampling sites. Figure 3 complements this by presenting box-and-whisker plots for each parameter across Captive (CV, Restrained), Captive (DCV, Restrained), Wild (CV, Restrained), and Wild (DCV, Restrained) groups. Significant differences ($p < 0.05$) were observed in 12 of 29 parameters between wild and captive sharks: RBC, PO, Alb, K, Mg, and Lip were significantly higher in wild sharks, whereas ALP, CPK, TG, Cre, P, and Glu were significantly higher in captive sharks. Significant differences ($p < 0.05$) between the CV and DCV samples occurred in 11 of 29 parameters: WBC, RBC, Ht, ALP, TP, Alb, TG, T-Cho, and K were significantly higher in CV samples, whereas CPK and BUN were significantly higher in

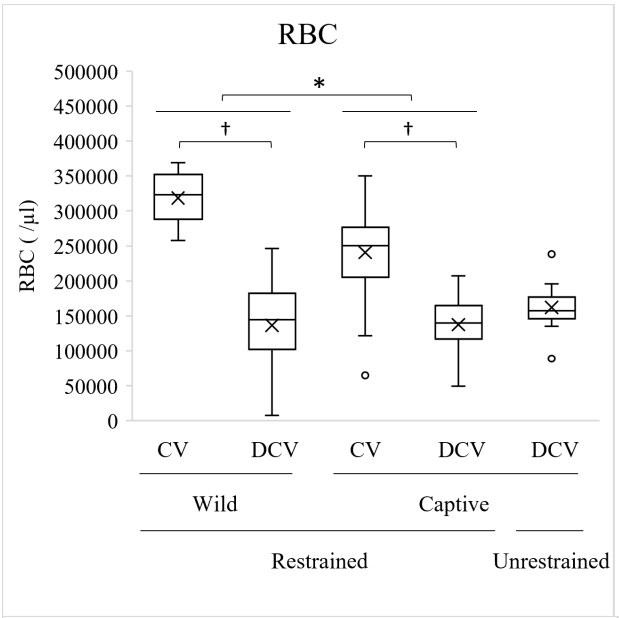
DCV samples. No significant interaction was observed between captivity status and blood sampling sites across all parameters. The detailed results of the two-way ANOVA, including degrees of freedom, mean square, *F*-values, and *p*-values, are provided in table S1.

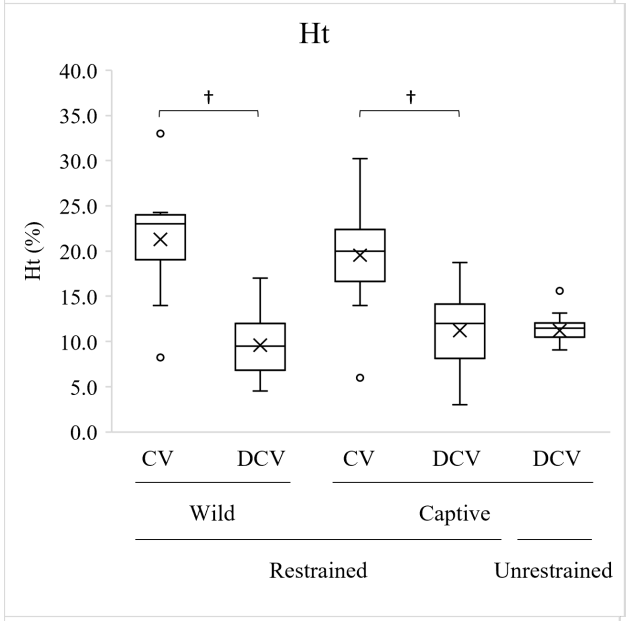
Table 2. Complete blood count and serum biochemical data and reference intervals (RIs) for restrained wild and captive whale sharks (*Rhincodon typus*)

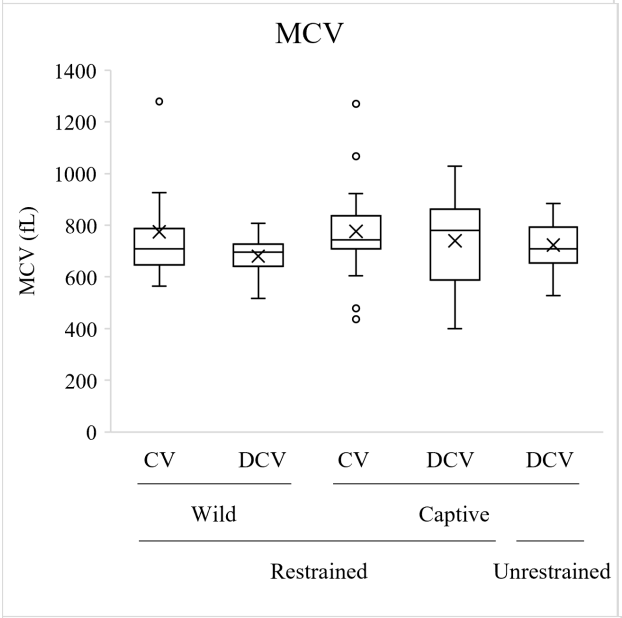
Parameter	Captivity Status	Blood Sampling Sites	Unit	Descriptive Statistics						Reference Interval with 90% Confidence Interval (CI)			Distribution	Method
				N	Mean	Median	SD	Min	Max	LCI	RI	UCI		
WBC	All	CV	/μl	29	11026	9600	5425	3500	24000	ND–4174	3027–24539	20168–29489	G	PT
	All	DCV	/μl	33	6955	7000	3503	670	14000	30–1908	773–15127	12864–17561	G	PT
RBC	Wild	CV	/μl	9	318350	323500	40413	258000	369000					
	Wild	DCV	/μl	15	136217	144750	65365	7400	246500					
	Captive	CV	/μl	20	240716	250500	69502	65000	350000	ND–135831	52376–361725	329877–392131	G	PT
	Captive	DCV	/μl	18	137511	139458	40620	49300	207000					
Ht	All	CV	%	30	20.7	20.5	5.7	6.0	33.0	2.9–11.4	7.5–31.1	28.6–33.6	G	PT
	All	DCV	%	35	10.5	10.3	4.1	3.0	21.5	0.6–4.4	2.5–18.1	16.3–20.1	G	PT
MCV	All	All	fL	49	742.6	720.3	178.5	400.0	1279.1	407.6–494.7	449.5–1169.6	1060.2–1294.2	G	PT
PO	Wild	All	mOsm/L	47	989.7	995.0	42.4	861.5	1172.0	876.8–945.8	910.1–1082.7	1050.1–1110.5	G	R
	Captive	All	mOsm/L	43	965.6	965.5	44.1	837.0	1065.8	836.1–891.2	864.7–1044.7	1029.8–1057.8	G	PT
AST	All	All	U/l	84	9.3	10.0	7.0	0.0	26.0	0.0–0.0	0.0–24.4	19.8–26.0	NG	NP
ALT	All	All	U/l	84	6.1	8.0	5.2	0.0	18.5	0.0–0.0	0.0–16.9	13.0–18.5	NG	NP
ALP	Wild	CV	U/l	18	21.2	19.5	11.9	1.0	47.0					
	Wild	DCV	U/l	27	14.5	17.0	10.3	1.0	35.0	0.0–0.0	0–36.8	29.9–42.7	NG	R
	Captive	CV	U/l	20	31.0	33.0	14.4	11.0	69.5	0–10.2	1.6–58.8	49.3–69.0	G	PT
	Captive	DCV	U/l	19	22.3	20.0	11.5	1.0	43.0					
LDH	All	All	U/l	82	63.6	53.8	47.9	0.0	206.0	0–5.0	1.1–180.6	150.0–206.0	NG	NP
GGT	All	All	U/l	82	1.9	1.0	2.3	0.0	10.0	0.0–0.0	0.0–7.0	7.0–8.6	NG	NP
T-Bil	All	All	mg/dl	81	0.11	0.10	0.05	0.00	0.20	0.0–0.0	0.0–0.2	0.2–0.2	NG	NP
TP	All	CV	g/dl	33	1.48	1.50	0.49	0.33	2.30	0.00–0.67	0.33–2.37	2.18–2.55	G	PT
	All	DCV	g/dl	30	0.96	0.94	0.29	0.40	1.60	0.35–0.56	0.45–1.64	1.44–1.86	G	PT
Alb	Wild	CV	g/dl	14	0.88	0.98	0.22	0.40	1.15					
	Wild	DCV	g/dl	16	0.67	0.70	0.14	0.30	0.90					
	Captive	CV	g/dl	18	0.73	0.70	0.18	0.30	1.20					
	Captive	DCV	g/dl	14	0.46	0.45	0.13	0.20	0.75					
NH_3	All	All	μg/dl	50	172.6	127.5	140.3	38.0	764.0	38.0–49.7	39.7–712.1	394.4–764.0	NG	NP
CPK	Wild	CV	U/l	16	48.0	47.8	28.7	0.0	112.0					
	Wild	DCV	U/l	26	88.8	83.5	40.6	17.5	190.0	6.5–33.7	18.5–184.6	154.2–218.2	G	PT
	Captive	CV	U/l	21	84.7	58.5	61.2	9.0	233.3	4.2–17.8	8.8–289.8	185.2–402.8	G	PT
	Captive	DCV	U/l	19	116.1	107.5	78.0	4.0	298.5					
TG	Wild	CV	mg/dl	18	10.0	9.8	8.5	0.0	27.0					
	Wild	DCV	mg/dl	27	3.4	2.0	3.5	0.0	12.5	NE	0–10.5	NE	NG	R
	Captive	CV	mg/dl	21	14.2	13.8	8.0	0.0	26.0	0–1.9	0–31.5	26.1–36.7	G	P
	Captive	DCV	mg/dl	20	6.2	2.4	6.8	0.0	24.0	NE	0–16.5	NE	NG	R
T-Cho	All	CV	mg/dl	39	30.1	27.3	19.7	0.5	87.5	0.0–4.5	1.3–75.7	62.4–90.6	G	PT
	All	DCV	mg/dl	47	14.3	13.5	9.9	0.5	40.0	0.5–5.0	1.0–39.2	32.0–40.0	NG	NP
BUN	All	CV	mg/dl	38	922.6	881.5	145.9	650.0	1410.0	678.2–748.8	711.0–1204.8	1113.4–1312.6	G	PT
	All	DCV	mg/dl	46	979.3	937.0	162.3	756.0	1500.0	710.0–773.9	738.9–1413.3	1264.1–1627.0	G	PT
UA	All	All	mg/dl	84	0.33	0.20	0.22	0.00	0.90	0.00–0.10	0.10–0.80	0.70–0.90	NG	NP
Cre	Wild	All	mg/dl	37	0.12	0.10	0.13	0.00	0.50	NE	NE	NE		
	Captive	All	mg/dl	40	0.30	0.22	0.27	0.01	0.80	0.01–0.01	0.01–0.80	0.80–0.80	NG	NP
Na	All	All	mEq/l	83	279.4	279.0	11.7	249.0	321.0	254.5–260.7	257.6–304.7	300.3–309.5	G	PT
K	Wild	CV	mEq/l	18	5.7	5.2	1.4	4.1	9.0					
	Wild	DCV	mEq/l	26	5.1	5.2	0.6	3.9	6.6	3.6–4.2	3.9–6.5	6.1–6.9	G	PT
	Captive	CV	mEq/l	21	4.9	5.0	0.8	3.4	7.0	2.9–3.7	3.3–7.0	6.3–7.8	G	PT
	Captive	DCV	mEq/l	20	4.5	4.4	1.0	3.2	7.5	2.9–3.4	3.1–7.9	6.2–11.6	G	PT
Cl	All	All	mEq/l	79	227.0	228.0	11.7	204.0	252.0	201.1–207.9	204.4–250.3	246.7–253.8	G	P
Ca	All	All	mg/dl	82	14.8	14.4	2.1	10.9	20.1	11.0–11.8	11.4–19.0	18.2–19.9	G	PT
iP	Wild	All	mg/dl	45	1.9	1.9	1.0	0.3	4.1	0.0–0.2	0.0–4.0	3.6–4.4	G	P
	Captive	All	mg/dl	41	2.6	2.8	0.8	0.8	3.8	0.8–1.4	1.1–4.2	3.9–4.6	G	P
Mg	Wild	All	mg/dl	25	4.0	4.0	0.7	3.1	5.8	2.2–3.0	2.6–5.5	5.1–5.9	G	P
	Captive	All	mg/dl	25	3.2	3.2	0.4	2.4	4.0	2.1–2.6	2.3–4.1	3.8–4.3	G	P
Glu	Wild	All	mg/dl	49	41.1	42.5	8.2	28.0	56.5	22.4–28.4	25.4–58.7	54.8–62.7	G	PT
	Captive	All	mg/dl	41	46.2	47.0	6.4	33.5	60.0	32.1–36.8	34.4–60.4	57.1–64.0	G	PT
Amy	All	All	U/l	84	2.6	3.0	1.9	0.0	7.0	0.0–0.0	0.0–7.0	6.0–7.0	NG	NP
Lip	Wild	All	U/l	23	42.4	41.0	4.4	37.0	54.5	28.1–35.8	31.2–50.6	46.2–54.0	G	R
	Captive	All	U/l	25	37.1	37.5	3.0	32.0	42.0	29.2–32.5	30.8–43.4	41.6–45.1	G	P

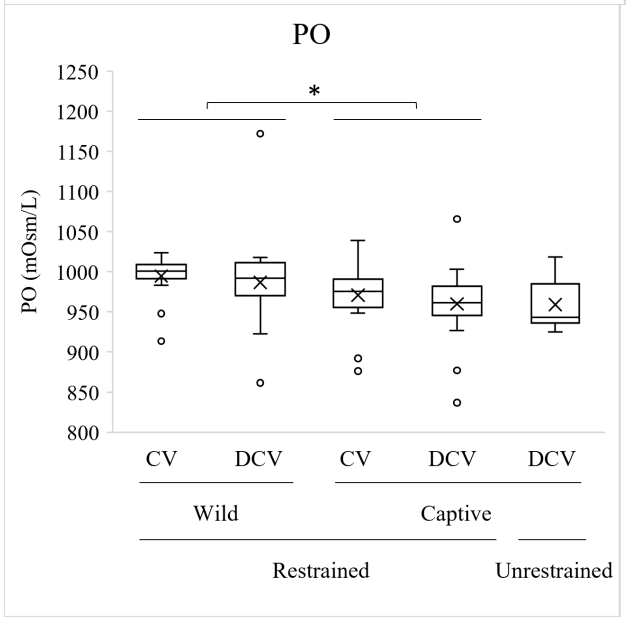
Regarding captivity status, "Wild," "Captive," and "All" indicate the data of restrained wild sharks, restrained captive sharks, and restrained both sharks, respectively. Regarding blood sampling site, "CV," "DCV," and "All" indicate the data of the caudal vein, dorsal cutaneous vein, and both veins of restrained sharks, respectively. N indicates number of individuals; SD, standard deviation; Min, minimum; Max, maximum; LCI, lower 90% confidence limit; UCl, upper 90% confidence limit; G, Gaussian; NG, non-Gaussian; P, parametric method; PT, parametric method with Box–Cox transformation; R, robust method; RT, robust method with Box–Cox transformation; NP, nonparametric; NE, variable could not be estimated; ND, no data.

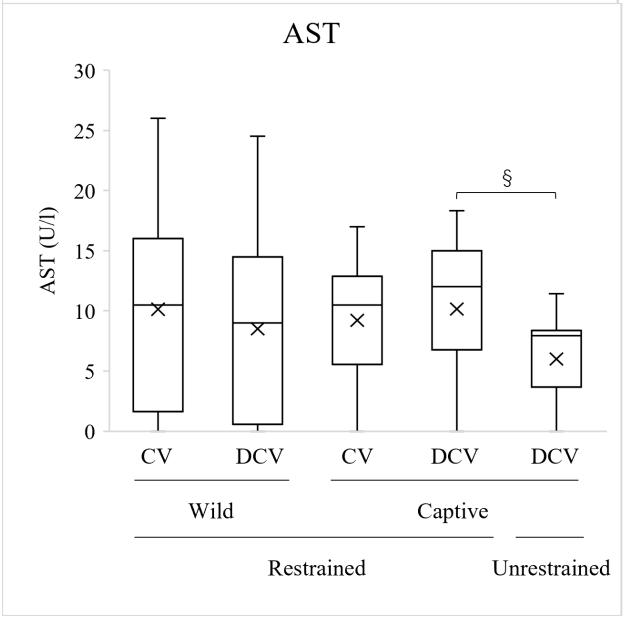

WBC
WBC (/µl)
30000
25000
20000
15000
10000
5000
0
†
†
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

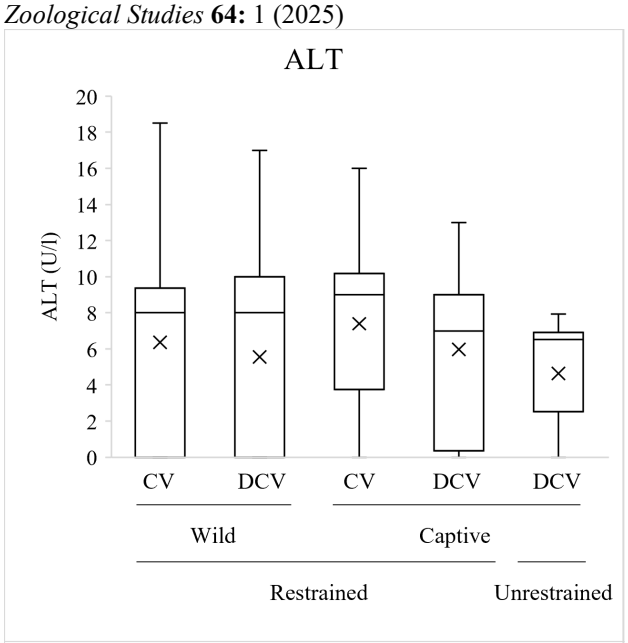

RBC
RBC (/µl)
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
*
†
†
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

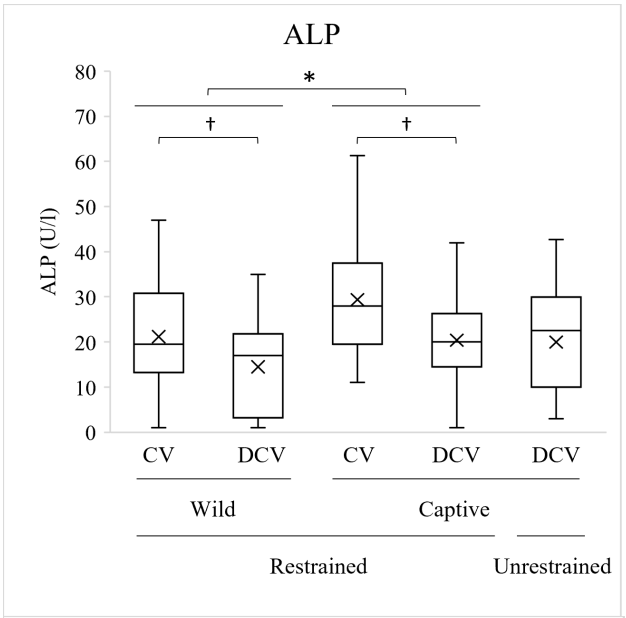

Ht
Ht (%)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
†
†
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

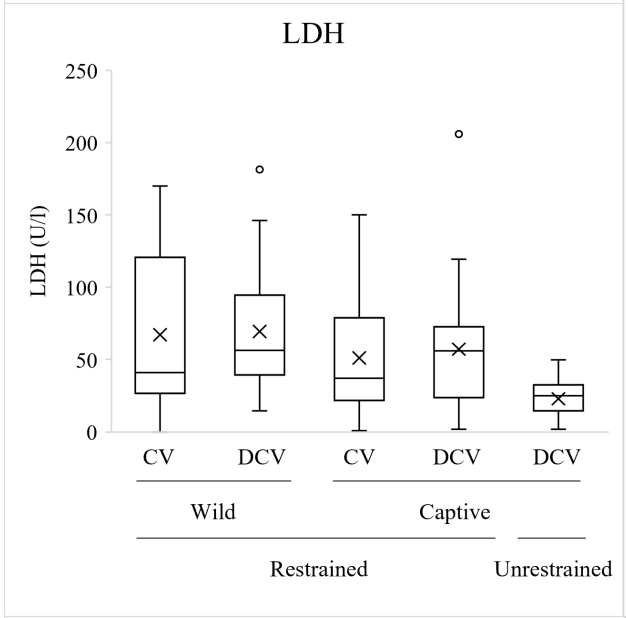

MCV
MCV (fL)
1400
1200
1000
800
600
400
200
0
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

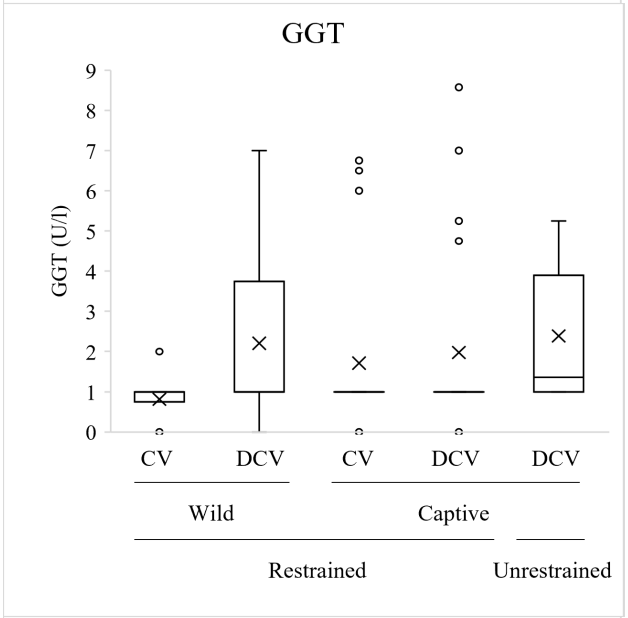

PO
PO (mOsm/L)
1250
1200
1150
1100
1050
1000
950
900
850
800
*
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

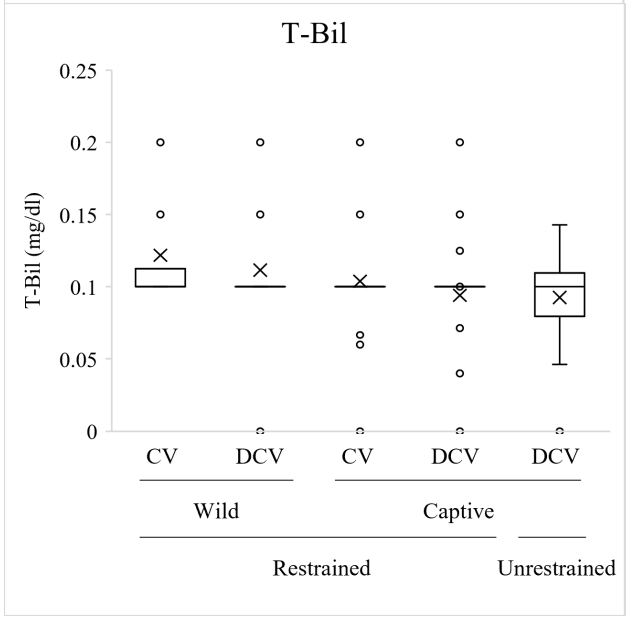

AST
AST (U/l)
30
25
20
15
10
5
0
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

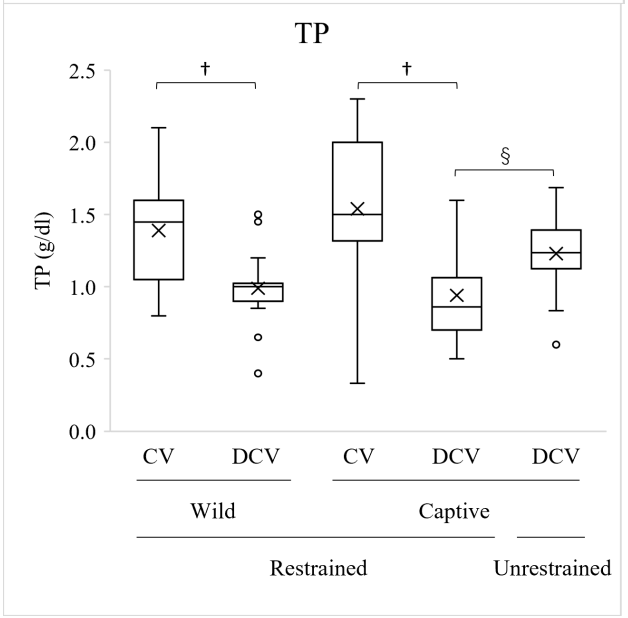

ALT
ALT (U/l)
0
2
4
6
8
10
12
14
16
18
20
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

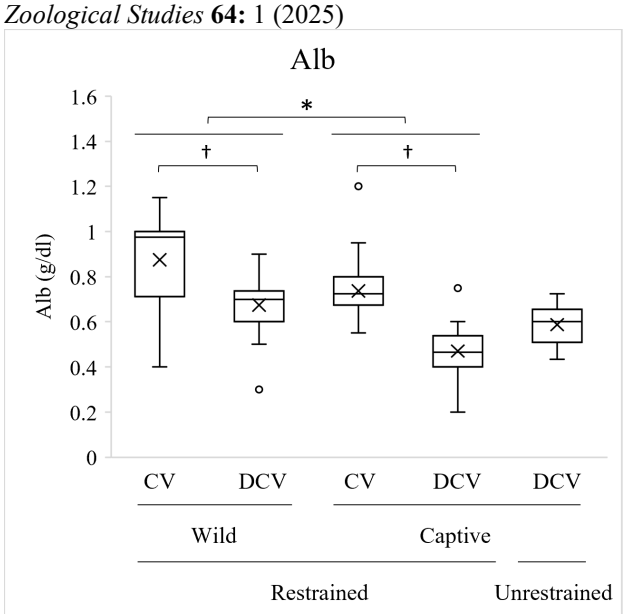

ALP
ALP (U/l)
0
10
20
30
40
50
60
70
80
*
†
†
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

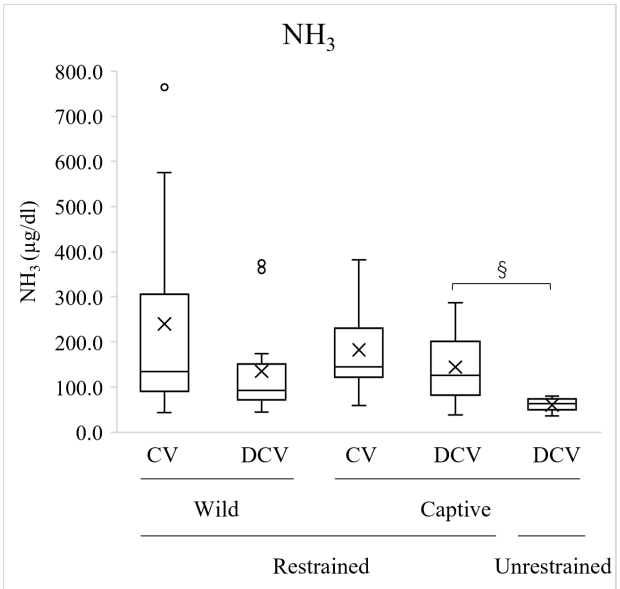

LDH
LDH (U/l)
0
50
100
150
200
250
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

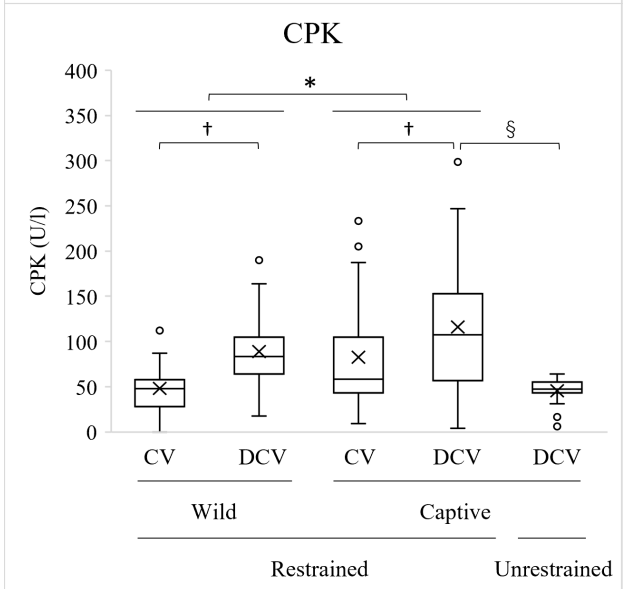

GGT
GGT (U/l)
0
1
2
3
4
5
6
7
8
9
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

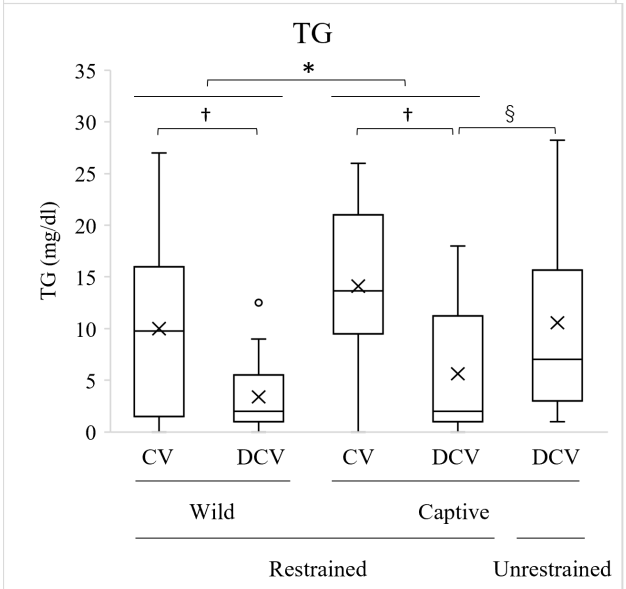

T-Bil
T-Bil (mg/dl)
0
0.05
0.1
0.15
0.2
0.25
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

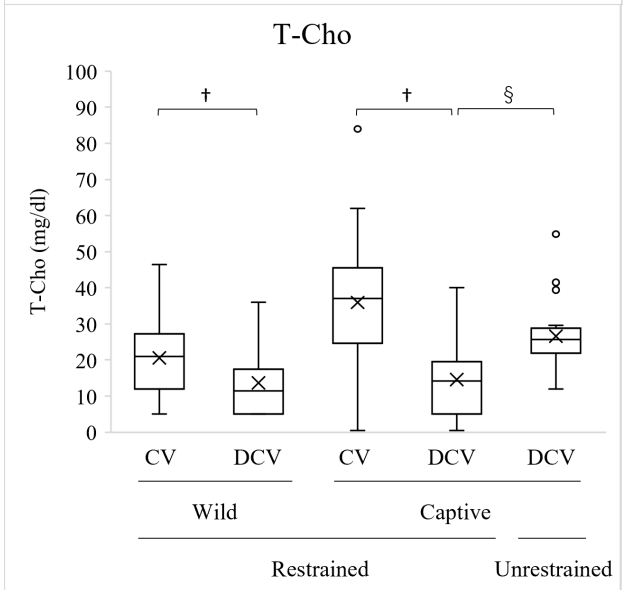

TP
TP (g/dl)
0.0
0.5
1.0
1.5
2.0
2.5
†
†
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

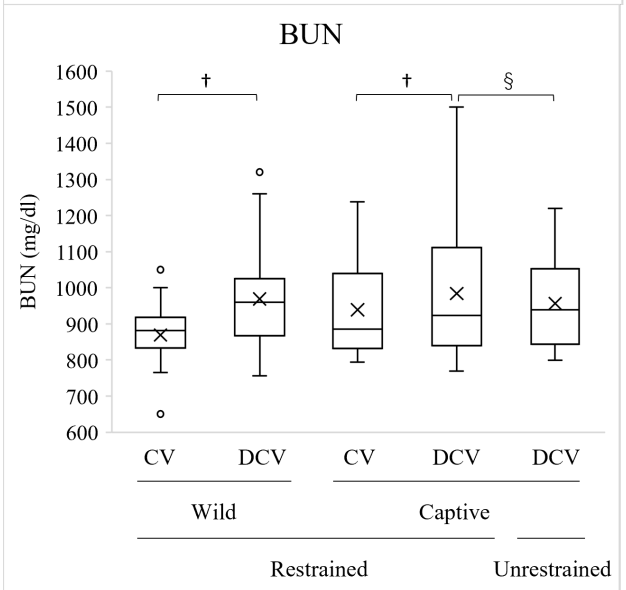

Alb
Alb (g/dl)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
†
†
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

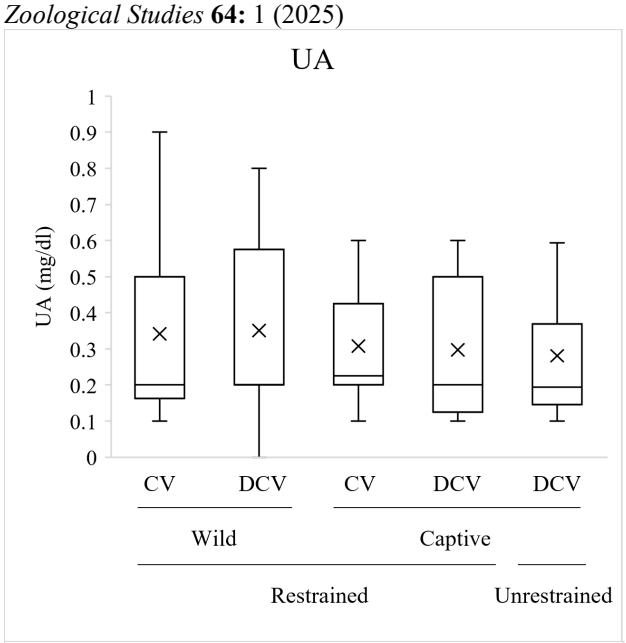

NH3
NH3 (μg/dl)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

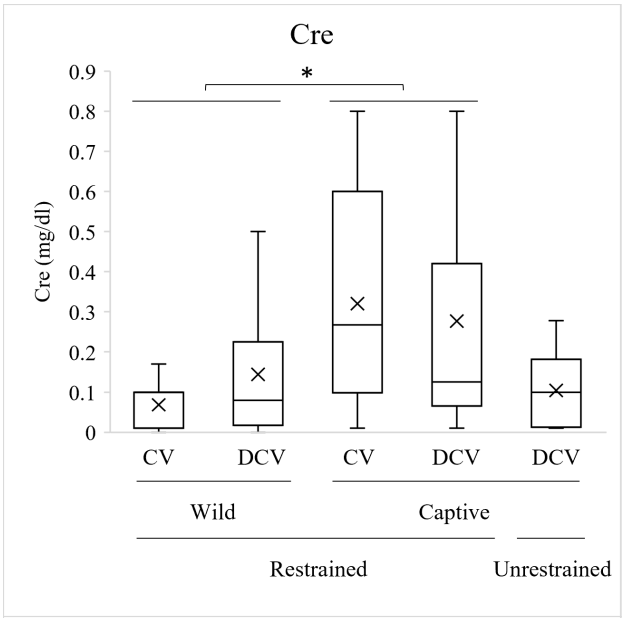

CPK
CPK (U/l)
400
350
300
250
200
150
100
50
0
*
†
†
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

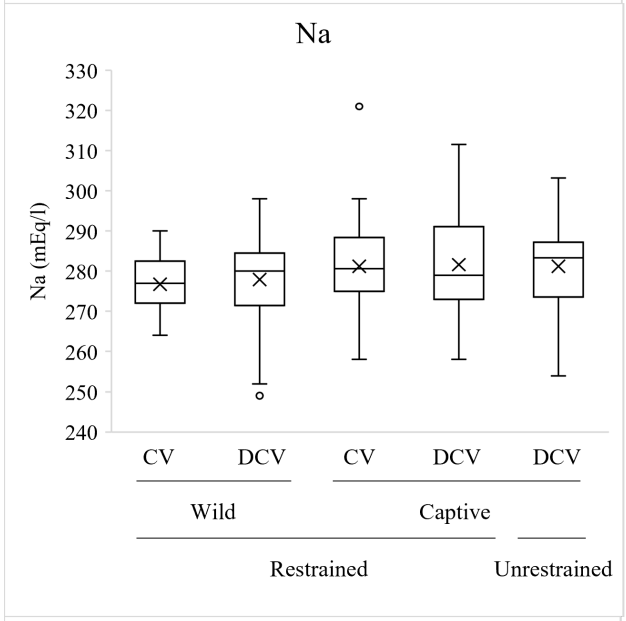

TG
TG (mg/dl)
35
30
25
20
15
10
5
0
*
†
†
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

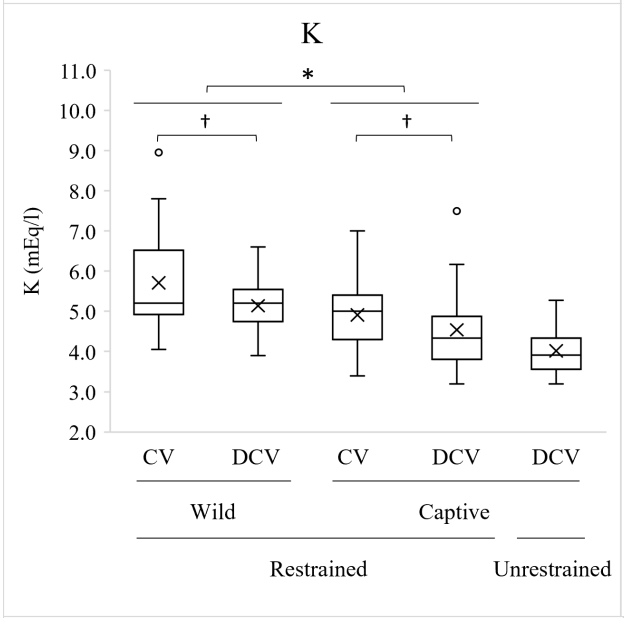

T-Cho
T-Cho (mg/dl)
100
90
80
70
60
50
40
30
20
10
0
†
†
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

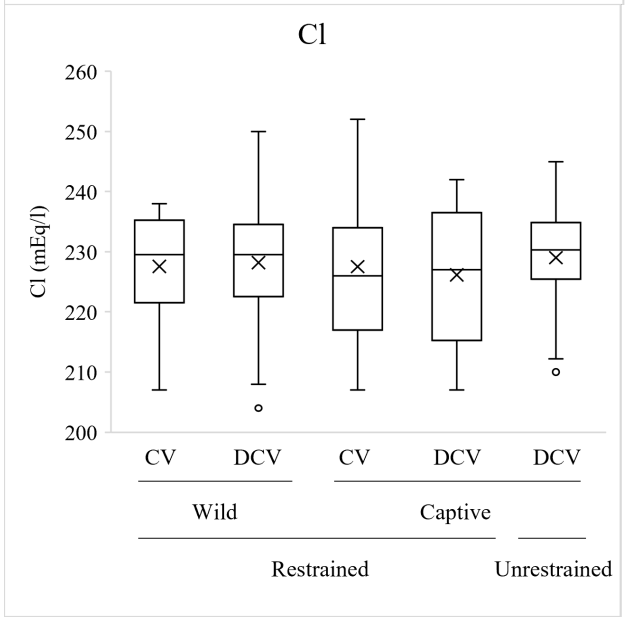

BUN
BUN (mg/dl)
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
†
†
§
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

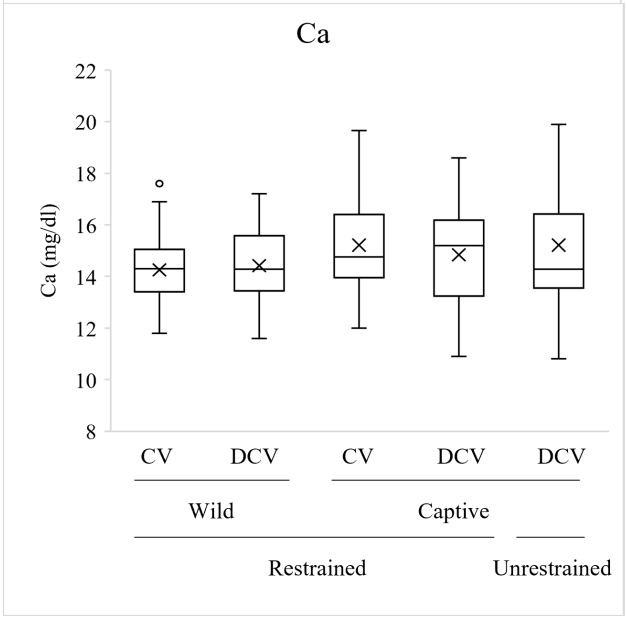

UA
UA (mg/dl)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained


Cre
Cre (mg/dl)
*
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained


Na
Na (mEq/l)
330
320
310
300
290
280
270
260
250
240
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained


K
K (mEq/l)
*
†
†
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained


Cl
Cl (mEq/l)
260
250
240
230
220
210
200
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained


Ca
Ca (mg/dl)
22
20
18
16
14
12
10
8
CV
DCV
CV
DCV
DCV
Wild
Captive
Restrained
Unrestrained

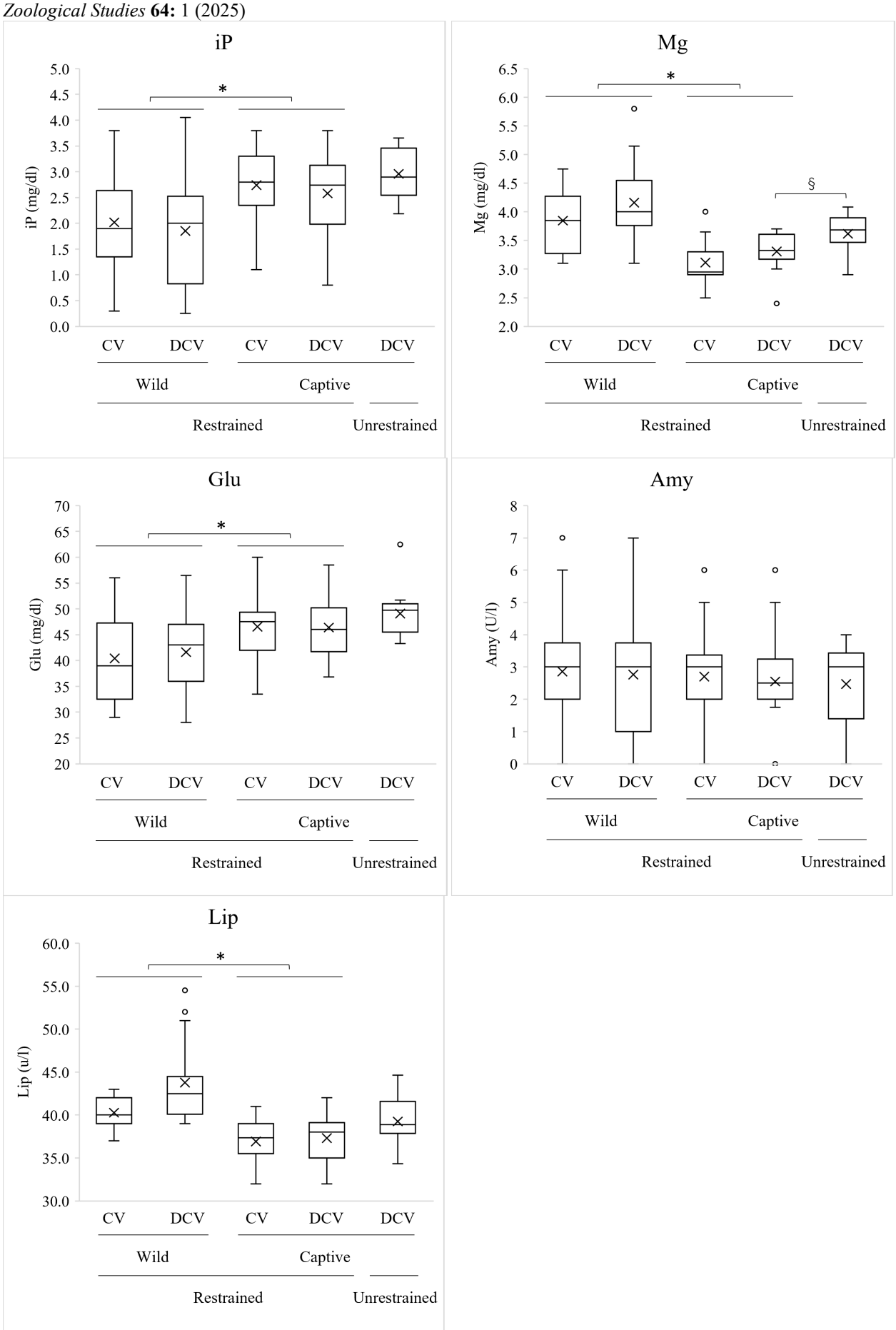


Fig 3. Box-and-whisker plots of 29 blood parameters for the groups Wild (CV, Restrained), Wild (DCV, Restrained), Captive (CV, Restrained), Captive (DCV, Restrained), and Captive (DCV, Unrestrained), categorized by captivity status, blood sampling site, and handling method. “Wild” and “Captive” represent data from wild and captive sharks, respectively. “CV” and “DCV” indicate data from the caudal vein and dorsal cutaneous vein, respectively. “Restrained”

and "Unrestrained" denote data from restrained and unrestrained sharks, respectively. The bottom and top of each box represent the first quartile and third quartile, respectively, with the line inside the box indicating the median. Whiskers extend to the smallest and largest values within 1.5 times the interquartile range from the first and third quartiles. Data points outside this range are considered outliers and are represented as individual dots. The × symbol indicates the mean value for each group. Groups with significant differences ($p < 0.05$) in captivity status and blood sampling sites, based on two-way ANOVA, and in handling methods, based on Student's *t*-test, are marked as follows: * for captivity status, † for blood sampling sites, and § for handling methods.

Blood Parameter Correlations Between Blood Sampling Sites

Table 3 presents correlations between blood parameters from Captive (CV, Restrained), Wild (CV, Restrained), Captive (DCV, Restrained), and Wild (DCV, Restrained) sharks for the CV and DCV. Strong correlations ($r > 0.4$; $p < 0.05$) were observed for all parameters except MCV and PO. Moreover, AST, ALT, ALP, LDH, GGT, Alb, NH_3, BUN, UA, Cre, Na, K, Cl, Ca, P, Mg, Glu, Amy, and Lip showed extremely strong correlations ($r > 0.7$).

Table 3. Correlation coefficients of complete blood count and serum biochemistry parameters between the caudal vein and dorsal cutaneous vein in wild and captive whale sharks (*Rhincodon typus*) under restraint

Parameter	n	r	*p*-value	a	b	Method
WBC	25	0.619	<0.001	0.393	3164.6	P
RBC	25	0.495	0.010	0.305	63378	P
Ht	24	0.407	0.048	0.257	5.600	P
MCV	19	0.033	0.894	0.027	697.2	P
PO	20	0.211	0.371	0.170	602.9	S
AST	35	**0.834**	<0.001	0.881	1.401	S
ALT	35	**0.749**	<0.001	0.775	0.810	S
ALP	35	**0.930**	<0.001	0.691	0.474	P
LDH	34	**0.815**	<0.001	0.778	15.750	S
GGT	34	**0.711**	<0.001	1.037	0.317	S
T-Bil	33	0.633	<0.001	0.611	0.042	S
TP	26	0.639	0.001	0.467	0.244	P
Alb	25	**0.794**	0.003	0.663	0.036	P
NH_3	21	**0.908**	<0.001	0.715	15.890	S
CPK	32	0.501	0.004	0.633	62.122	S
TG	36	0.626	<0.001	0.403	0.198	S
T-Cho	36	0.484	0.003	0.279	6.304	S
BUN	34	**0.754**	<0.001	0.747	270.880	S
UA	35	**0.929**	<0.001	0.982	0.013	S
Cre	29	**0.854**	<0.001	0.816	0.009	S
Na	33	**0.760**	<0.001	0.684	89.730	P
K	37	**0.798**	<0.001	0.594	1.633	S
Cl	32	**0.793**	<0.001	0.789	48.576	P
Ca	34	**0.843**	<0.001	0.861	1.842	P
iP	36	**0.948**	<0.001	1.027	0.156	P
Mg	21	**0.769**	<0.001	0.800	0.866	P
Glu	38	**0.814**	<0.001	0.744	11.770	P
Amy	35	**0.851**	<0.001	0.935	0.235	S
Lip	20	**0.755**	<0.001	1.192	5.911	S

Bold indicates $r > 0.7$; a, the coefficient of a linear function; b, the intercept of a linear function; P, Pearson correlation coefficient; S, Spearman's rank correlation coefficient.

Comparison of Handling Methods

Table 4 provides descriptive statistics and RIs based on Student's *t*-test results comparing blood parameters between restrained and unrestrained conditions. Figure 3 complements this result

by presenting box-and-whisker plots for each parameter in Captive (DCV, Restrained) and Captive (DCV, Unrestrained) sharks. Eight parameters showed significant differences: AST, NH_3, CPK, and BUN showed significantly higher values ($p < 0.05$) under restraint, whereas TP, TG, T-Cho, and Mg exhibited significantly higher values ($p < 0.05$) under unrestrained conditions. The remaining parameters displayed no significant difference between the restrained and unrestrained conditions.

Table 4. Complete blood count and serum biochemical data and reference intervals (RIs) of captive whale sharks (*Rhincodon typus*) following dorsal cutaneous vein sampling

Parameter	Handling Methods	Unit	Descriptive Statistics						Reference Interval with 90% Confidence Interval (CI)			Distribution	Method
			N	Mean	Median	SD	Min	Max	LCI	RI	UCI		
WBC	All	/μl	34	8097	8382	2619	2500	14000	1517–4050	2775–13574	12227–14964	G	PT
RBC	All	/μl	34	148979	150628	38474	49300	238375	52307–87940	69561–228397	209060–246918	G	P
Ht	All	%	32	11.2	11.6	3.2	3.0	18.8	1.7–6.0	4.0–17.5	16.0–18.9	G	PT
MCV	All	fL	29	730.7	755.7	139.3	400.0	1027.8	372.2–513.2	440.4–1021.0	944.4–1094.3	G	PT
PO	All	mOsm/L	39	959.5	957.0	39.1	837.0	1065.8	856.3–903.6	880.1–1040.3	1014.4–1063.8	NG	R
AST	Restrained	U/l	19	10.2	12.0	6.1	0.0	18.3					
	Unrestrained	U/l	17	6.0	7.9	4.0	0.0	11.4					
ALT	All	U/l	36	5.3	6.8	3.8	0.0	13.0	0.0–0.3	0–14.1	12.4–15.6	G	R
ALP	All	U/l	36	20.2	20.0	11.1	1.0	42.7	0.0–2.5	0.0–43.0	37.6–48.2	G	P
LDH	All	U/l	35	40.5	29.0	40.2	2.0	206.0	0.8–4.3	1.9–162.3	112.2–221.0	G	PT
GGT	All	U/l	36	2.2	1.0	2.1	0.0	8.6	NE	0.0–4.3	NE	NG	R
T-Bil	All	mg/dl	36	0.09	0.10	0.04	0.00	0.20	NE	0.01–0.17	NE	G	RT
TP	Restrained	g/dl	15	0.94	0.86	0.31	0.50	1.60					
	Unrestrained	g/dl	17	1.23	1.24	0.28	0.60	1.69					
Alb	All	g/dl	31	0.53	0.51	0.12	0.20	0.75	0.22–0.34	0.28–0.79	0.72–0.85	G	P
NH_3	Restrained	μg/dl	12	144.4	125.9	80.8	38.0	287.0					
	Unrestrained	μg/dl	13	60.5	63.0	13.6	36.0	79.7					
CPK	Restrained	U/l	19	116.1	107.4	80.1	4.0	298.5					
	Unrestrained	U/l	17	45.6	47.5	15.6	6.0	64.0					
TG	Restrained	mg/dl	20	5.6	2.0	6.2	0.0	18.0	NE	0.0–16.5	NE	NG	R
	Unrestrained	mg/dl	17	10.6	7.0	8.4	1.0	28.2					
T-Cho	Restrained	mg/dl	20	14.6	14.3	11.2	0.5	40.0	0.0–2.0	0.4–49.5	34.1–69.3	G	PT
	Unrestrained	mg/dl	17	26.6	25.7	10.7	12.0	54.9					
BUN	Restrained	mg/dl	19	983.9	924.0	185.6	769.0	1500.0					
	Unrestrained	mg/dl	17	956.7	939.1	126.9	799.8	1220.0					
UA	All	mg/dl	36	0.29	0.20	0.18	0.10	0.60	0.00–0.00	0.00–0.67	0.53–0.77	NG	R
Cre	All	mg/dl	36	0.19	0.10	0.22	0.01	0.80	0.00–0.00	0.00–0.99	0.59–1.66	G	PT
Na	All	mEq/l	37	281.4	280.0	12.4	254.0	311.5	250.5–261.5	255.9–307.0	301.0–312.7	G	P
K	All	mEq/l	37	4.3	4.2	0.9	3.2	7.5	3.0–3.3	3.2–6.6	5.8–7.8	G	PT
Cl	All	mEq/l	37	227.5	229.9	10.7	207.0	244.9	200.8–210.3	205.4–249.5	244.4–254.5	G	P
Ca	All	mg/dl	36	15.0	15.0	2.4	10.8	19.9	9.1–11.2	10.1–19.9	18.7–21.0	G	P
iP	All	mg/dl	37	2.8	2.8	0.7	0.8	3.8	1.0–1.6	1.3–4.2	3.9–4.5	G	P
Mg	Restrained	mg/dl	12	3.3	3.3	0.4	2.4	3.7					
	Unrestrained	mg/dl	13	3.6	3.7	0.3	2.9	4.1					
Glu	All	mg/dl	37	47.6	47.0	5.6	36.8	62.5	33.8–38.7	36.2–59.1	56.4–61.6	G	P
Amy	All	U/l	36	2.5	2.8	1.4	0.0	6.0	0–0.2	0–5.4	4.8–6.1	G	P
Lip	All	U/l	25	38.3	38.5	3.0	32.0	44.6	30.3–33.7	31.9–44.7	42.9–46.4	G	P

Regarding handling methods, "Restrained," "Unrestrained," and "All" indicate the data of the captive restrained sharks, captive unrestrained sharks, and captive both sharks, respectively. N indicates number of individuals; SD, standard deviation; Min, minimum; Max, maximum; LCI, lower 90% confidence limit; UCl, upper 90% confidence limit; G, Gaussian; NG, non-Gaussian; P, parametric method; PT, parametric method with Box–Cox transformation; R, robust method; RT, robust method with Box–Cox transformation; NP, nonparametric; NE, variable could not be estimated; ND, no data.

DISCUSSION

Based on blood samples collected from the CV and DCV of whale sharks under restraint, this study revealed significant differences in 12 parameters between wild and captive individuals. Additionally, significant differences were observed in 11 parameters between CV and DCV samples from both wild and captive restrained sharks, as well as significant correlations for all parameters except MCV and PO. Among captive sharks subjected to DCV sampling, eight blood data parameters differed significantly under restrained and unrestrained conditions. These results provide baseline RIs for blood parameters in whale sharks, calculated within single or combined factor levels.

Prior studies reported differences in blood data between wild and captive sharks of various elasmobranch species, with whale sharks specifically studied by Ueda et al. (2017). Their study which compared 9 parameters revealed significant differences in T-Cho, TG, and BUN values, all of which were higher in wild sharks, but no significant difference in Glu levels. In contrast, our analysis revealed a significant difference in Glu levels but no significant differences in T-Cho and BUN values. Considering that both their whale sharks and ours were captured in Japanese waters, these discrepancies may be attributed to differences in husbandry environments between theirs and ours.

The differences observed between wild and captive sharks may be due to various environmental factors, with nutritional differences being the most plausible explanation. Shark diets are typically characterized by high protein, high fatty acid, and low carbohydrate content (Speers-Roesch 2010). A substantial portion of total liver lipid content in sharks comprises TGs, which serve as their primary energy source (Gallagher et al. 2017; James 1997). Research on tiger sharks (*Galeocerdo cuvier*) (Gallagher et al. 2014b) has shown that blood TG concentrations are lower in lean sharks and higher in their obese counterparts, with a significant correlation existing between TG levels and body condition, highlighting blood TG as a potential shark health indicator. Additionally, a study on great white sharks (*Carcharodon carcharias*) revealed decreases in liver lipid reserves during migration, impacting buoyancy (Del Raye et al. 2013). Similarly, observations of wild whale sharks off the Okinawa coast, approximately 1,000 km southwest of Kochi, showed low TG values in four of eight sharks, indicative of prolonged fasting periods lasting up to four months (Wyatt et al. 2019). These findings align with the low TG values observed in wild sharks in the present study. Limited data on whale shark migration to Japanese waters, as reported by Matsunaga et al. (2003), suggest that they migrate northward along the Kuroshio current from tropical regions in search of prey around spring. They temporarily reside in the Kuroshio-Oyashio region (approximately 1,000 km northeast of Kochi), where prey resources are abundant in the warm waters, before departing from Japanese waters in autumn as sea temperatures decline. This implies that whale sharks migrating to the Kuroshio-Oyashio region during spring–summer likely experience extended periods of limited feeding along both the Okinawa and Kochi coasts.

RBC counts were higher in wild sharks than in captive sharks; however, no significant differences in Ht and MCV values were observed, suggesting no logical correlation among these three parameters. In a study measuring manual complete blood counts in sandbar sharks (*Carcharhinus plumbeus*) under various conditions, Arnold (2005) found that WBC, Ht, and Hb levels fell within acceptable error ranges for manual counting of human cells, whereas RBC counts exceeded this range. Consequently, manual RBC counts in elasmobranchs are considered estimates, and diagnostic assessments, such as anemia tests, should rely on Ht or Hb values instead. Thus, the

observed discrepancies among RBC, Ht, and MCV values in whale sharks suggest that RBC and MCV findings should be treated as estimates, with statistical results interpreted cautiously.

We found significant differences in 11 parameters between CV and DCV blood sampling in both wild and captive whale sharks under restrained conditions. To the best of our knowledge, only four studies have compared CV and DCV sampling in sharks, including whale sharks. Two studies focused on species other than whale sharks (Mylniczenko et al. 2006; Naples et al. 2012), comparing Ht, blood gas parameter, Glu, and lactate values among multiple species, finding that CV sampling yielded significantly higher values for all parameters. For whale sharks, a nonstatistical comparison was conducted using data from two individuals and four samples, covering Ht, WBCs, TP, and lactate; results revealed a tendency for CV values to be higher across all parameters (Dove et al. 2010). Another study on whale sharks found statistically significant differences in blood parameters taken from the pectoral fin, first dorsal fin, and second dorsal fin, although specific parameters were not mentioned (Wyatt et al. 2019). In our study, among the 11 parameters exhibiting significant differences, CPK and BUN values were unexpectedly higher in DCV samples. CPK in elasmobranchs is often considered a marker for liver and skeletal muscle function (Wosnick et al. 2017; Starostinetsky-Malonek et al. 2023; Cliff and Thurman 1984; Otway 2015), whereas BUN is influenced by factors such as nutritional status (Morón-Elorza et al. 2022; Takahashi et al. 2014), stress (Ruiz-Jarabo et al. 2022; Morón-Elorza et al. 2022; Starostinetsky-Malonek et al. 2023), and environmental water quality and salinity (Grant et al. 2020). However, the mechanism underlying the elevated values in DCV samples from whale sharks remains unclear.

For 19 parameters showing a strong correlation ($r > 0.7$) between CV and DCV samples, linear regression could be used to predict CV sampling values based on DCV sampling data. However, no correlation was found for MCV and PO. The lack of correlation in MCV may be due to the inaccuracies previously discussed. In contrast, the lack of correlation in PO may be related to the PVS and the SVS in fish. Unlike terrestrial vertebrates, fish do not possess a lymphatic system. Teleosts have an SVS that connects to the PVS through contractile muscles, which contribute to filtering most RBCs and WBCs from the PVS, allowing only plasma to pass into the SVS. Consequently, the complete blood counts of the SVS and PVS differ (Satchell 1999). Sharks are believed to possess a similar system, with the CV and DCV corresponding to the PVS and SVS, respectively (Naples et al. 2012). In teleosts, the SVS has various functions, including nutrient delivery to the skin, cutaneous respiration, cardiac workload reduction, RBC reduction, and PO and ion buffering in the PVS (Rummer et al. 2014; Satchell 1999). A more detailed understanding of the SVS's roles in elasmobranchs, particularly regarding its contribution to PO buffering, could help clarify the lack of correlation in PO observed in this study.

In captive sharks, significant differences were found in eight parameters between blood

data collected via DCV sampling under restrained and unrestrained conditions. Although this comparison used only DCV data, the high correlation between CV and DCV samples suggests that similar results would likely be observed following CV sampling. Establishing Ris for blood parameters in fish requires considering the impact of restraint stress on biochemical values. Methods allowing quick and efficient restraint and sampling (Otway 2015; Cliff and Thurman 1984) or chemical sedation (Otway et al. 2011) help minimize stress, but completely eliminating the impact of stress remains challenging (Brown 1993). In the present study, we used a method for blood sampling without restraint, believed to reduce human-induced stress, by applying training techniques based on behavioral analysis theory, similar to those used in marine mammal management (Brando 2010). Under restrained conditions, AST, NH_3, CPK, and BUN values were significantly elevated. Numerous studies have examined stress in elasmobranchs, especially in sharks, in relation to catch-and-release practices in fisheries and leisure activities (Moyes et al. 2006; Mandelman and Skomal 2009; Gallagher et al. 2014a). Based on these studies, AST and CPK levels likely increased due to muscle damage caused by restraint (Stoskopf 1993; Otway et al. 2011; Wells et al. 1986; Stoskopf 2010; Harms et al. 2002; Cain et al. 2004; Cliff and Thurman 1984; Manire et al. 2001). NH_3 levels are also known to rise after intense physical activity (Gudrun et al. 2015). However, although BUN levels typically decline under stress conditions (Ruiz-Jarabo et al. 2022; Morón-Elorza et al. 2022; Starostinetsky-Malonek et al. 2023), BUN was unexpectedly elevated in our study. Significantly lower values were observed for TP, TG, T-Cho, and Mg under restraint. As TP, TG, and T-Cho are nutritional indices, these findings likely reflect energy consumption caused by restraint stress. To the best of our knowledge, blood Mg levels in elasmobranchs have not been reported previously. However, in marine teleost fishes, concentrations of monovalent ions, such as Na and Cl, and divalent ions, such as Mg and Ca, are affected by acute stress (Skomal and Mandelman 2012); thus, a similar mechanism may exist in whale sharks. Although leukocytosis has been reported in stressed elasmobranchs (Arnold and Delaune 2022), it was not observed in our restrained whale sharks. Further research is needed to understand how the type, duration, and intensity of stress affect blood stress parameters in whale sharks.

Based on our statistical results, we propose Ris calculated for single or combined levels within each factor. Although blood parameter reference values have been reported for captive whale sharks based on descriptive statistics (Dove et al. 2010 2022; Ueda et al. 2017), differences are expected in our study owing to variations in blood analysis methods, tank size, water quality, diet, tank social structure, visitor presence, and shark origin. Additionally, we used mean values for each shark, rather than all data, reducing the total number of samples and preventing Ris from being calculated for some parameters. A minimum of 120 samples from 120 healthy individuals is recommended for determining Ris with a 90% confidence interval using non-Gaussian methods

(Friedrichs et al. 2012). Increasing the number of sharks sampled in future studies is necessary to accurately determine more Ris and understand their physiological and clinical significance, which is essential for managing both captive and wild populations.

CONCLUSIONS

Our analysis of blood parameters in both wild and captive whale sharks revealed significant differences in multiple parameters based on captivity status, blood sampling sites, and handling methods. RIs were established considering these factors, offering new insights not reported in previous studies. These RIs are valuable for detecting health issues early through fluctuations in blood parameter levels in captive sharks, enabling prompt management responses during practices such as capture, transportation, and handling. Comparing these results to parameters related to wild sharks' behavior and ecology, along with associated environmental factors, may improve our understanding of wild population physiology and contribute to conservation efforts through health risk management. Future studies that collect blood data from adequately feeding whale sharks in the Kuroshio-Oyashio region, and compare these with the data from captive and wild sharks in this study, could further enhance our knowledge of captive management and wild population ecology.

List of abbreviations

Alb, Albumin.

ALP, Alkaline phosphatase.

ALT, Alanine aminotransferase.

Amy, Amylase.

AST, Aspartate aminotransferase.

BUN, Blood urea nitrogen.

Ca, Calcium.

Cl, Chloride.

Cre, Creatinine.

CPK, Creatine phosphokinase.

CV, Caudal vein.

DCV, Dorsal cutaneous vein.

GGT, γ-glutamyltransferase.

Glu, Glucose.

Ht, Hematocrit.

iP, Inorganic phosphate.

K, Potassium.

LDH, Lactate dehydrogenase.

Lip, Lipase.

MCV, Mean corpuscular volume.

Mg, Magnesium.

Na, Sodium.

NH_3, Ammonia.

OBIC, Osaka Aquarium Biological Research Institute of Iburi Center.

PO, Plasma osmolality.

PVS, Primary vascular system.

RBC, Red blood cell.

RI, Reference interval.

SVS, Secondary vascular system.

T-Bil, Total bilirubin.

T-Cho, Total cholesterol.

TG, Triglyceride.

TP, Total protein.

UA, Uric acid.

WBC, White blood cell.

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Supplementary materials

Table S1. Results of Two-Way ANOVA for Captivity Status and Blood Sampling Site, Including Degrees of Freedom (Df), Mean Squares (MS), *F*-values, and *p*-values