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REFERENCE INTERVALS

Establishment of preliminary reference intervals and cytochemical staining of blood cells in big-bellied seahorses (*Hippocampus abdominalis*)

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Abstract

Background: Despite their popularity, hematology reference intervals (RIs) have not been established in big-bellied seahorses (*Hippocampus abdominalis*).

Objective: The objectives of this study were to establish hematologic RIs to compare values between sex in regard to cytochemical staining of blood cells. We also sought to compare white blood cell concentrations using the Natt and Herrick technique vs blood smear estimates.

Methods: Forty-three healthy individuals from the Aquarium du Québec (22 females and 21 males) were included. Normal health status was confirmed by an unremarkable physical examination in five individuals and by necropsy of five other individuals, of which all were excluded from further analyses. Venipuncture was performed from the ventral coccygeal vein in the remaining 33 individuals without anesthesia using heparinized insulin syringes. A blood volume of 0.05 to 0.1 ml was collected to prepare Wright Giemsa-stained blood smears and hematocrits immediately after venipuncture. Whole blood was stored in heparinized Eppendorf tubes to determine red and white blood cell concentrations using the Natt and Herrick technique with a hemocytometer in 10 individuals; these results were compared with blood smear estimates. Additional blood smears were stained with alkaline phosphatase substrate, periodic acid Schiff, and toluidine blue stains.

Results: The reference intervals included the packed cell volume (27.4–67.5%), thrombocyte count (19.5–197.7 $\times 10^9$/L), and white blood cell (WBC) count (2–54.8 $\times 10^9$/L), including neutrophils (1.1–21.3 $\times 10^9$/L), lymphocytes (2.7–45.5 $\times 10^9$/L), and monocytes (0–2.2 $\times 10^9$/L). The WBC hemocytometer counts showed no correlation with blood smear estimates (Spearman's rho = 0.2). There was also no significant difference between the sexes.

Conclusions: These preliminary reference intervals will help assess the health of seahorses.

KEYWORDS
blood smear, coccygeal vein, hematology, syngnathidae

1 | INTRODUCTION

Big-bellied seahorses (*Hippocampus abdominalis*) are one of the most prevalent seahorses in zoological institutions worldwide due to their large size and relative ease of reproduction in captivity.[1] In addition, seahorses are commonly bred in aquaculture in Asia for use in Chinese medicine.[2] While at least 16 out of 42 species of seahorses were considered threatened at the time of publication, as determined by the International Union for Conservation of Nature,[3] no hematology reference intervals (RIs) have been described in any seahorse species to date.

Hematology is a clinical test commonly used in public aquaria, and RIs have been established in various fish species, such as the koi (*Cyprinus carpio*)[4] and cownose ray (*Rhinoptera bonasus*), among others.[5] Hematologic measurements have been shown to vary with inflammation and infection in fish, as reported in other vertebrate species.[6,7] In particular, mycobacteriosis is one of the three most common causes of mortality in captive seahorses.[8] In lined sea horses (*Hippocampus erectus*), stress has been associated with heterophilia and an increase in the heterophil to lymphocyte ratio,[9] although hematology RIs and cell morphology were not described.

The objectives of this study were to establish hematologic RIs in male and female seahorses and compare the RI values between the sexes. We also sought to characterize cytochemical staining of blood cells and compared white blood cell (WBC) concentrations measured with the Natt and Herrick technique vs those estimated on a blood smear.

2 | MATERIAL AND METHODS

This research project was approved by the Institutional Animal Care and Use Committee of the Faculté de médecine vétérinaire, Université de Montréal (20-Rech-2090, approved on Nov 1st 2021).

2.1 | Reference population

Forty-three 6- to 8-month-old big-bellied seahorses from the Aquarium du Québec (22 females and 21 males) were included in the study. These individuals were bred on site. Seahorses were maintained in saltwater (Instant Ocean Salt, Instant Ocean, Blacksburg, VA) in four tanks kept at 16°C and equipped with standard filtration systems (Fluval, Rolf C. Hagen Inc, Baie-D'Urfé, QC, Canada) and ultraviolet light for disinfection. Weekly water quality checks were carried out in each tank, including total ammonia, pH, nitrite, nitrates, and alkalinity; results remained within normal limits during the study. Prey was offered three times daily and included thawed and live artemia (Hikari Bio-pure Brine-Shrimp), gut-loaded with a fatty acid supplement (Selco DHA, INVE Aquaculture, Dendermonde, Belgium), and copepods (Calanus Piscine Energetics Inc, Vernon, BC, Canada).

2.1.1 | Determination of health

Normal health status was confirmed by a 6-month clinical follow-up and included the unremarkable physical examination of five randomly selected seahorses anesthetized with tricaine methanesulfonate (MS-222, Aqualife TMS, Syndel, Nanaimo, BC, Canada) and five other necropsied individuals that had originated from all tanks. Briefly, five seahorses were gently caught with an atraumatic net and transferred to a water bucket containing 20 mg/L of MS-222 and an air stone. They were weighed (range 6-16 grams), visually examined, sexed, and identified with a colored elastomer (Visible Implant Elastomer, Northwest Marine Technology, Inc, Shaw Island, WA) using a predetermined code. A skin scrape was obtained and examined under the microscope. These individuals were not used for blood collection. Five other individuals were gently caught and transferred to an aerated water bucket containing 500 mg/L of MS-222. After cessation of respiratory movements, each individual was pithed, weighed, and the coelom was incised. Each seahorse was fixed whole in neutral-buffered 10% formalin. Tissues (brain, heart, gills, hepatopancreas, spleen, kidneys, gonads, digestive tract, skin, and muscle) were routinely processed and stained with H&E and saffron staining for histopathologic examination. Then, Ziehl Neelsen staining was carried out on all tissues submitted to rule out mycobacteriosis. Staining with Periodic Acid Schiff (PAS), with and without amylase, was carried out thereafter on renal tissue and splenic. Histologic evaluation of the tissues was performed by the same operator (JR), while slides of renal tissue were also evaluated by a board-certified pathologist (MOBB).

2.1.2 | Exclusion criteria

Male seahorses were excluded from the study if they were visibly pregnant, that is, they carried eggs or fry in their pouch.[10] In addition, individuals were excluded from the study if any abnormality was detected on initial, visual, and physical examination (eg, skin parasites). None of the individuals from the study met these criteria; thus, none were excluded.

2.1.3 | Animal follow-up

Each individual that had venipuncture was followed for at least 6 months afterward, and any adverse effects were recorded.

2.2 | Sample handling and analysis

2.2.1 | Sample collection

Each of the 33 remaining seahorses was transferred to a 2-liter bucket of water without an anesthetic agent equipped with an air stone. Venipuncture (Figure 1) was performed on awake seahorses with a pre-heparinized insulin syringe in the ventral coccygeal vein using a

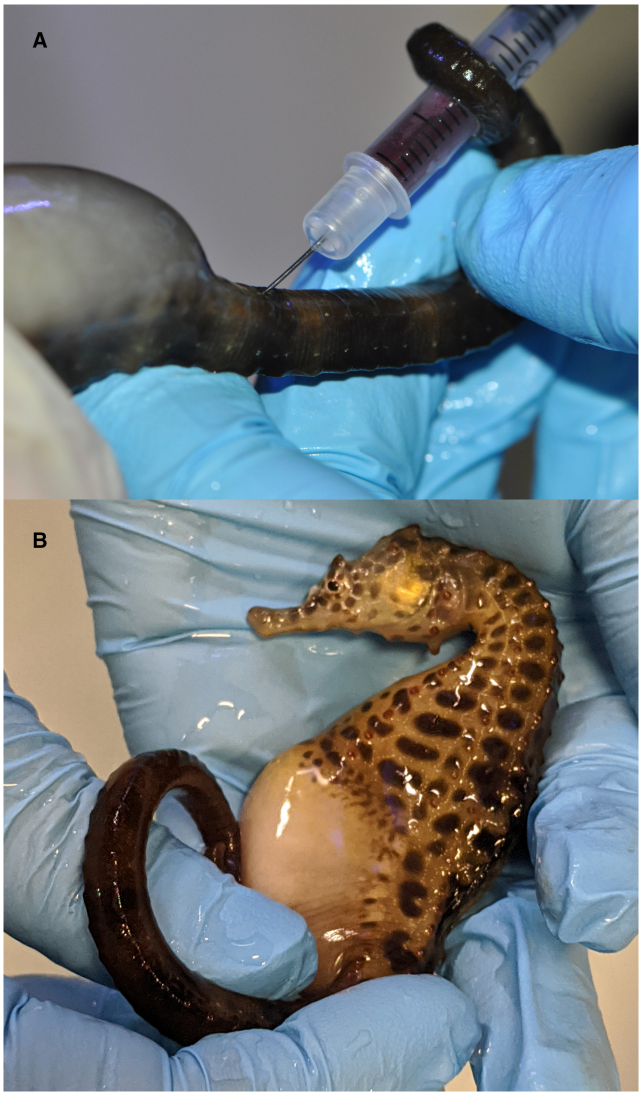

FIGURE 1 (A) Venipuncture of the ventral coccygeal vein using a ventral midline approach in a big-bellied seahorse. Cranial is toward the left, and caudal is towards the right of the image. (B) Example of tail discoloration distally to the venipuncture site.

ventral approach, keeping the head immersed in water. Since a pilot study had shown signs of hemolysis on blood smears when MS-222 was used, venipuncture was performed awake. A maximum blood volume corresponding to 1% of the body weight was collected (range: 0.06–0.16 mL). After blood collection in the insulin syringe, the top of the syringe containing the needle was removed with a hemostat, and heparinized capillary tubes (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were filled. Six to eight blood smears were immediately prepared for each individual. The remaining blood of 10 randomly selected individuals was transferred to 250 µL Eppendorf vials containing 1–4 µL of heparin (10 000 UI/mL, Pfizer, Kirkland, QC, Canada).

2.2.2 | Sample handling and analysis

Packed cell volumes were measured following standard centrifugation (Centri-V 1586 Centrifuge, Leading Edge Veterinary Equipment, Springbrook, WI, USA) of a hematocrit capillary tube at 45875*g* for 120 seconds. Initially, Eppendorf vials were kept on ice for 3-hour transportation, which resulted in blood clotting of five samples. The protocol was adjusted to keep the Eppendorf vials at 25°C during transportation. Subsequently, no further clotting was noted.

Air-dried smears were stained with Wright Giemsa and special stains, including Alkaline Phosphatase substrate stain (ALP), also known as Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP), Periodic Acid Schiff (PAS) with and without amylase, and toluidine blue (n = 10 for each stain, randomly selected). Samples stored in Eppendorf vials enabled the determination of red blood cell (RBC) and WBC counts using the Natt and Herrick technique for 10 distinct individuals, as recommended in fish.[11] Briefly, 5 µL of heparinized blood were drawn using a pipette, added to 995 µL of the Natt and Herrick stain (dilution factor 1:200), and thoroughly mixed. The mixture was allowed to sit for 5 minutes at 22°C for optimal staining and was then transferred to each chamber of a Neubauer-ruled hemacytometer by capillary action. After loading, the sample sat for 1–2 additional minutes more. RBCs were counted in the large central and four corner squares with a x40 objective in each chamber. The average number of RBCs in both chambers was calculated and then multiplied by 10 000 to obtain the total number of RBCs $\times 10^9$/L. Then, WBCs were counted in the nine large squares in each chamber of the hemocytometer with a ×10 objective. The average of both chambers was used for the WBC calculation. White blood cell counts were determined using the following formula:

$$\text{WBC count}\left(10^9/\text{L}\right) = (\text{total count in 9 squares}\,(\text{mean of both chambers}) + 10\%) \times 200.$$[11]

White blood cell differential counts were established by counting 100 WBCs. Thrombocytes were not determined using this method.

White blood cell counts were estimated in 10 fields with a x40 objective on Wright Giemsa-stained smears. The average of the 10 fields was then multiplied by a correction factor of 1.57 for our microscope, as established in our laboratory. The field number of the x40 objective is 22, and the field of view is 22/400 = 0.055. A WBC differential count was established by a single operator (laboratory technician) using a 100 WBC count, including neutrophils, lymphocytes, and monocytes. Red blood cell and WBC morphologies were evaluated by a single operator according to the standard operating procedure in the laboratory. To grade polychromatophilia, a semi-quantitative evaluation was performed; 2-10 polychromatophils per field were graded as 1+, 11-14 as 2+, 15-30 as 3+, and >30 as 4+.[12] Thrombocytes were calculated as the mean of 10 fields (×100 objective) and multiplied by 20 (microscope adjustment factor) to give the number of thrombocytes $\times 10^9$/L. The field of view for this calculation was 22/1000 = 0.022.

White blood cells were identified based on their morphologic characteristics on Wright Giemsa-stained blood smears with a x50 immersion objective. Additional blood slides were evaluated for

each animal using special stains to verify cell tinctorial properties. These included the ALP substrate, PAS with and without amylase, and toluidine blue stains. Positive controls were used for each stain. The staining intensity of special stains was graded with a semi-quantitative scale, including three levels: positive, partially positive, and negative. Sections of the anterior kidney and spleen of necropsied individuals were further stained with PAS with and without amylase to evaluate lymphohematopoietic organs, as these are the main hematopoietic organs in fish.

2.3 | Statistical analysis

Reference intervals were established following the American Society for Veterinary Clinical Pathology guidelines for zoological species.[13] Results were computed using the Reference Value Advisor software (v2.1). Data were examined graphically using side-by-side boxplots as recommended for small sample sizes.[13] Outliers were detected graphically using the Tukey technique in normally distributed measurands and excluded. The Shapiro–Wilk test was used to determine data normality with a *P*-value of 0.19, as recommended.[14] Reference limits were calculated using a parametric approach when normally distributed, initially or after a Box-Cox transformation, and a robust method when data were non-normally distributed. In addition, 90% upper and lower confidence intervals were established for each reference limit

Data were then analyzed using statistical analysis software (R version 3.4.1, R Core Team [2021]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org). Because data were not normally distributed, the non-parametric Spearman correlation test was used to assess the correlation between the different methods to estimate WBC counts. Values of $\rho \geq 0.9$ were considered to demonstrate an excellent correlation (weak when $\rho < 0.4$, moderate when $0.4 \leq \rho < 0.7$, and strong when $0.7 \leq \rho < 0.9$). Non-normally distributed data were reported as the range (median). Mann–Whitney tests were carried out to compare thrombocyte counts and absolute WBC counts between male and female seahorses. Values of $P < 0.05$ were considered significant for this analysis.

3 | RESULTS

The absence of parasites was confirmed from the skin scrapes in all seahorses examined beforehand. All seahorses recovered from venipuncture and retained a normal appetite. The only noted adverse effect was a temporary discoloration of the distal part of the tail in some individuals (Figure 1).

Reference intervals determined using WBC concentration estimates on blood smears are reported in Table 1 and Figure 2. No reliable RIs could be established for monocyte percentages due to a low prevalence. Basophils and eosinophils were not observed. Regarding cell morphology, slight anisocytosis of red blood cells was noted on all blood smears. Poikilocytosis was absent. Polychromatophilia of 1+ intensity was noted in 94% of blood smears. There was an absence of reactive lymphocytes and toxic change in the neutrophils. Occasional polyhedric inclusions were noted in thrombocytes (Figure 3). No significant difference was noted between males and females; thus, global RIs are reported

Regarding cell tinctorial properties, Figure 3 and Table 2 display the results of each special stain.

Using the Natt and Herrick technique, WBC counts ranged from 38 720 x10^9/L to 946 125 x10^9/L (median: 162000 x10^9/L). No correlation was noted between WBC count estimates and the results from the Natt and Herrick technique (Spearman correlation coefficient $\rho = 0.21$, $P = 0.56$). The evaluation of cells using the Natt and Herrick technique was very challenging as ballooning of all cells was noted, and the intensity of the pink staining in WBC was very variable over time (staining intensity decreasing in WBCs and increasing in RBCs after approximately 10 minutes).

In addition, on histology, myeloid precursors displayed mildly positive staining for PAS and PAS with amylase, while erythroid precursors were negative for PAS and PAS with amylase in the anterior kidney (Figure 4).

TABLE 1 Hematology reference interval established in aquarium-housed big-bellied seahorses using white blood cell concentrations estimated from Wright Giemsa-stained blood smears

Measurand	Units	n	Min-Max	Mean	Median	Reference interval	90% CI lower	90% CI upper	Method
Packed Cell Volume [a]	%	27	33-65	47.4	45.0	27.4-67.5	22.5-32.6	62-72.7	P
Thrombocytes[a]	10^9/L	27	22-182	108.6	110.0	19.5-197.7	0-42.7	173.3-221.0	P
White blood cells	10^9/L	32	4.7-51.4		30.6	1.2-55.0	−4.6-7.5	47.8-61.1	R
Neutrophils[a]	10^9/L	32	0.5-20.8	3.7	3.3	1.1-21.3	0.3-2.3	16.9-25.3	BC+P
Lymphocytes[a]	10^9/L	32	2.8-50.0	5.8	6.1	2.7-45.5	1.6-4.7	36.8-55.3	BC+P
Monocytes	10^9/L	33	0-2.1		0.9	0-2.3	−0.4-0	2.0-2.7	R
Neutrophil: lymphocyte ratio		29	0.19-1.06		0.5	0-1	−0.2-0.1	0.8-1.1	R

Abbreviations: BC, Box Cox transformed; CI, Confidence interval; n, number of individuals; P, parametric; R, robust.

[a]Normal distribution with a *P*-value >0.19 (after box-cox transformation when indicated in the last column).

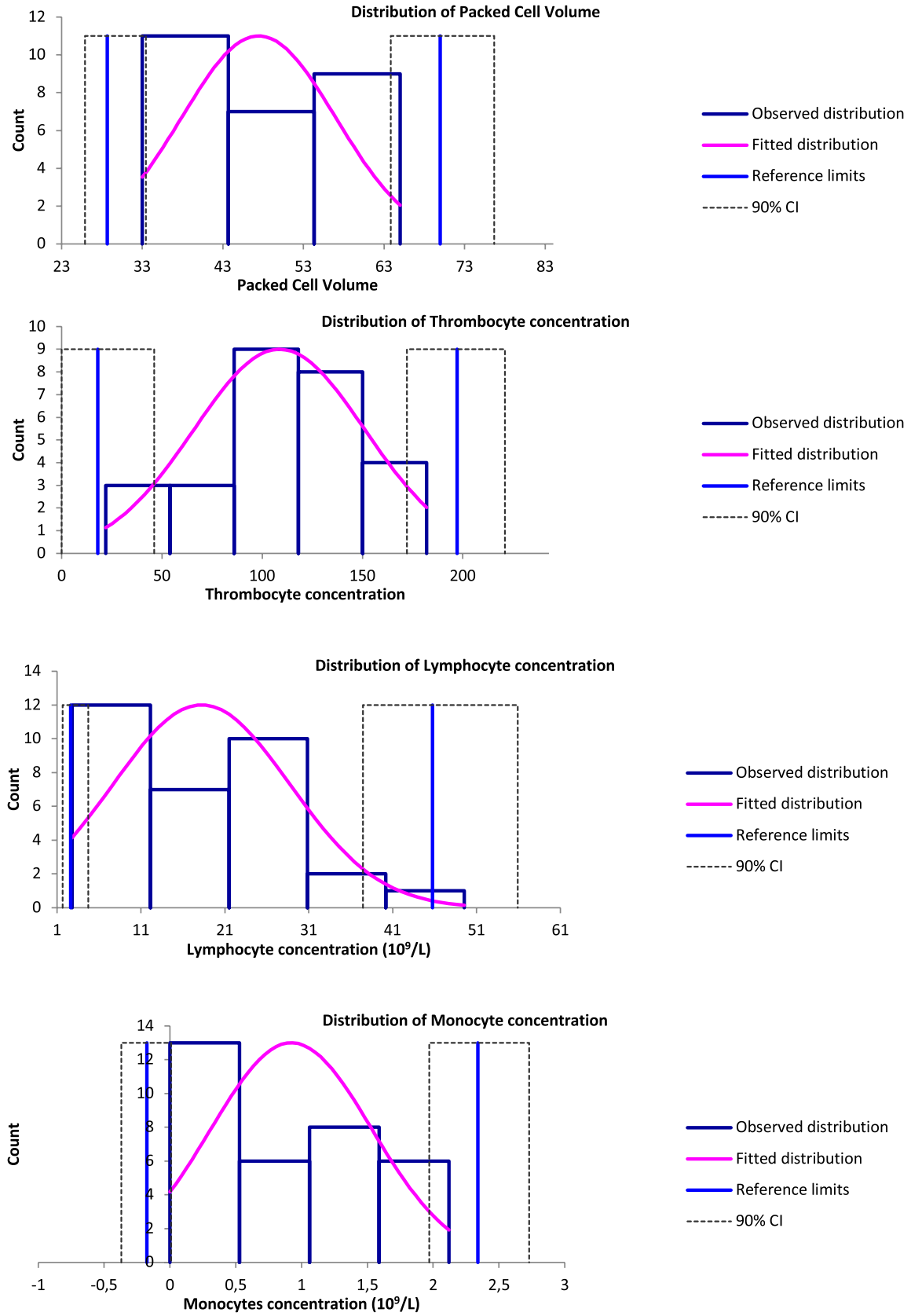

FIGURE 2 Distribution histograms for the packed cell volumes, neutrophil to lymphocyte ratios, and thrombocyte, white blood cell, neutrophil, lymphocyte, and monocyte counts in aquarium-housed big-bellied seahorses.

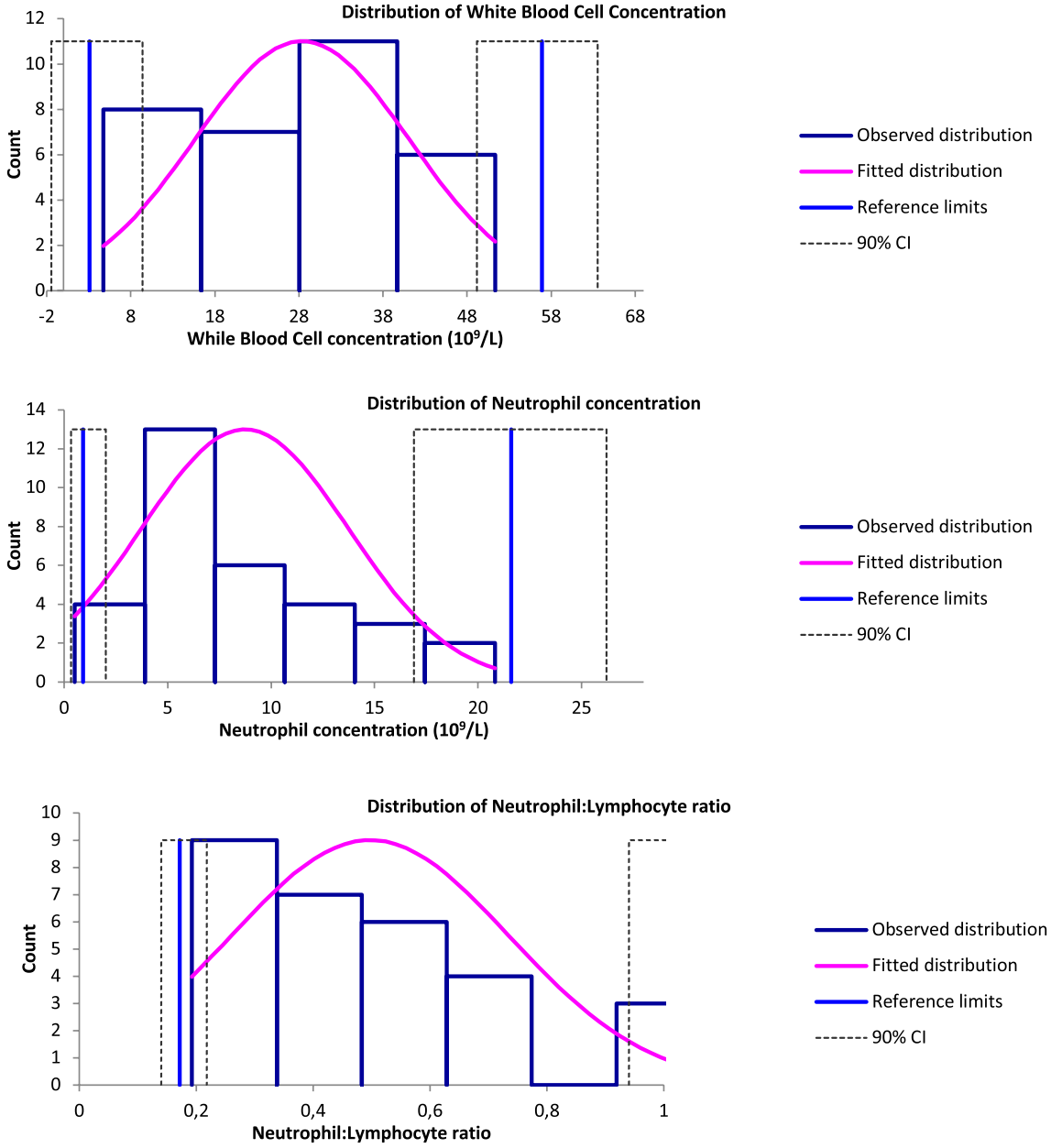

FIGURE 2 (Continued)

4 | DISCUSSION

In this study, preliminary hematology RIs were established in big-bellied seahorses. Results were highly variable among individuals, resulting in wide RIs. Packed cell volume RIs were higher than previously reported in 16 lined seahorses, with a mean of 20.6% and a standard deviation of 5.1% in a previous study.[9] The wild-caught lined seahorses could have been anemic in the previous study due to stress and multiple infectious conditions detected on necropsy,[9] while the big-bellied seahorses of the present study were healthy and born in captivity. Lymphocytes represented the predominant WBC, as commonly reported in teleost fish,[15] including lined seahorses.[7]

Clinically, seahorses recovered well from the procedure. Venipuncture was initially performed under anesthesia with MS-222 during a pilot study, but this was associated with hemolysis of samples placed in heparin and ethylenediaminetetracetic acid. Thus, all subsequent venipunctures were performed in awake animals, and no hemolysis was subsequently noted. Transient tail discoloration could be due to a local neurologic lesion, as previously reported in chameleons undergoing ventral coccygeal vein venipuncture.[16] This adverse effect was transient, generally lasting a few weeks, and did not appear to impact the general health of affected animals as all seahorses were still able to cling to the decor and eat normally.

Five blood cell types could be differentiated with the special stains used in this study. In the present study, granulocytes were named neutrophils due to the absence of cytoplasmic granules (noted with the Wright Giemsa smears), while they were called heterophils in a previous study in lined seahorses.[9] Neutrophils were PAS positive in big-bellied seahorses as reported in koi and channel catfish (*Ictalurus punctatus*).[4,17] In contrast to koi neutrophils,

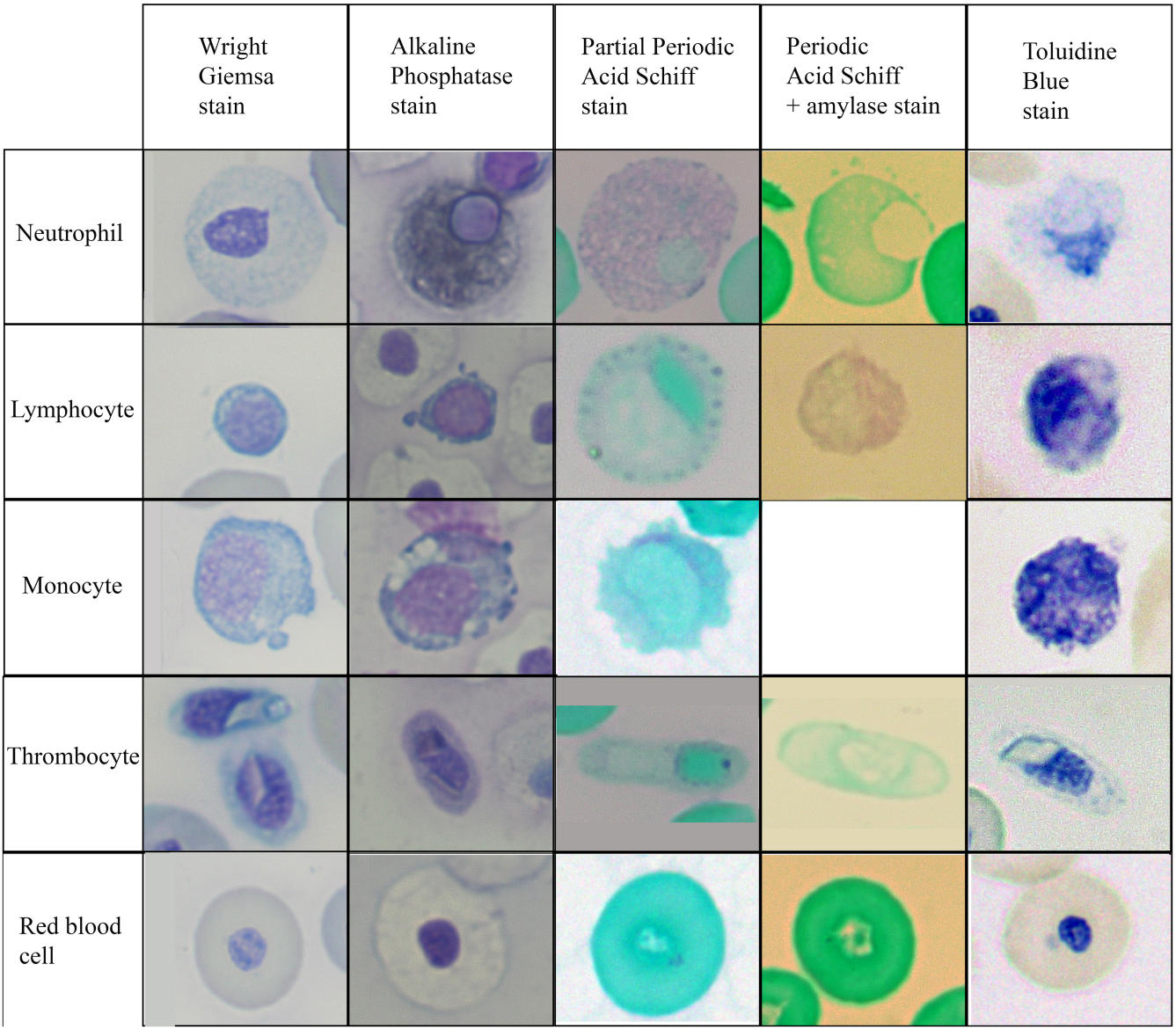

FIGURE 3 Photomicrographs of various blood cells in big-bellied seahorses using different staining methods listed at the top; ×40 objective.

TABLE 2 Cytochemical staining properties of big-bellied seahorse blood cells (n = 10 for each stain)

	ALP	PAS	PAS with amylase	Toluidine blue
Neutrophils	+	+	−	−
Lymphocytes	−	+/−	+/−	+
Monocytes	−	−	−	+
Thrombocytes	−	+/−	−	−
Red blood cells	−	−	−	−

Abbreviations: ALP, alkaline phosphatase; PAS, periodic acid schiff.

seahorse neutrophils also stained positively with ALP.[4] Toluidine blue staining distinguished positively staining lymphocytes from morphologically similar and negatively staining thrombocytes in big-bellied seahorses. In other species (eg, the diamondback rattlesnake), PAS with diastase/amylase has been shown useful in differentiating thrombocytes from lymphocytes as only thrombocyte cytoplasm was focally stained.[18] In the present study, staining was similar between lymphocytes and thrombocytes using PAS without amylase, while only lymphocytes were partially stained using PAS with amylase. Therefore, positive toluidine blue and/or PAS with amylase could be used in big-bellied seahorses to differentiate lymphocytes and thrombocytes. In catfish, basophils have been reported to stain positive with toluidine blue[17]; however, basophils were not observed in this study. The absence of eosinophils and basophils was not unexpected, as these cells have only been reported rarely in fish.[3,19] Overall, the observed variability observed among fish species highlights the need to conduct species-specific studies to characterize blood cell tinctorial properties in fish.

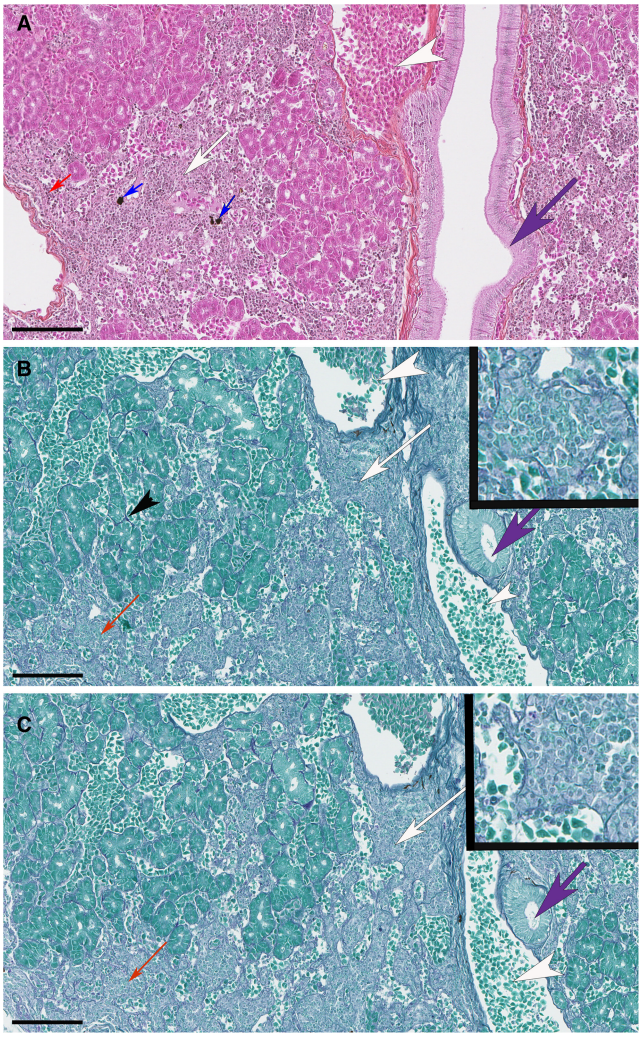

FIGURE 4 Anterior kidney of a big-bellied seahorse: (A) Hematoxylin phloxine saffron stain. (B) PAS stain (close-up: erythroid precursors). (C) PAS with amylase (inset: myeloid precursors) Bar = 100 µm. Positive structures are blue, while negative structures are green. White arrowheads point to red blood cells in a blood vessel, purple arrows point to renal tubules, blue arrows point to iron storage, the black arrowhead points to the PAS-positive basement membrane, white arrows point to myeloid precursors (mildly positive for PAS), and red arrows point to erythroid precursors (negative for PAS). Cell staining was not affected by pretreatment with amylase.

Technical challenges were noted with the Natt and Herrick technique. Initially, sample coagulation might have been due to the storage temperature, as increasing the amount of heparin did not resolve the issue, while increasing the temperature during transportation did. The observed cell ballooning might be explained by a high osmolarity of big-bellied seahorse blood, as reported in other fish living in saltwater. Since red and white blood cells are nucleated in seahorses, differentiating these cells with certainty was sometimes difficult given the ballooning artifacts. In sharks, an adjustment in the osmolarity of the sample is needed by adding salt and urea to the Natt and Herrick reagent.[20] It is unknown if such an adjustment would be beneficial in seahorses as blood osmolarity was not evaluated in this study or other studies of seahorses to the authors' knowledge. Furthermore, although WBCs were counted first to alleviate the issue of color intensity, high variability was still noted for this method. Further studies are needed to validate the use of the Natt and Herrick technique in seahorses. A limitation of the present study is the wide RI ranges observed in this small population, despite homogenous husbandry. This may have been due to the variability of the blood smear evaluation, although they were performed by one operator.

In summary, preliminary RIs were established in big-bellied seahorses, no sex-associated differences were noted in a limited number of individuals, blood cell cytochemical reactions were established, and the estimation of WBC counts on blood smears is recommended pending more studies evaluating the Natt and Herrick technique.

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DISCLOSURE

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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REFERENCES

1. Koldewey H, Mulligan M, Shedd J. *Syngnathid Husbandry in Public Aquariums - 2005 Manual*. ZSL; 2005:137.
2. Koldewey HJ, Martin-Smith KM. A global review of seahorse aquaculture. *Aquaculture*. 2010;302(3–4):131-152.
3. Zhang X, Vincent ACJ. Predicting distributions, habitat preferences and associated conservation implications for a genus of rare fishes, seahorse (*Hippocampus* spp.). *Divers Distrib*. 2018;24(7):1005-1017.
4. Tripathi NK, Latimer KS, Burnley VV. Hematologic reference intervals for koi (*Cyprinus carpio*), including blood cell morphology, cytochemistry, and ultrastructure. *Vet Clin Pathol*. 2004;33(2):74-83.
5. Ferreira CM, Field CL, Tuttle AD. Hematological and plasma biochemical parameters of aquarium-maintained cownose rays. *J Aquat Anim Health*. 2010;22(2):123-128.
6. Lu J, Lu H, Cao GJ. Hematological and histological changes in prussian carp *Carassius gibelio* infected with cyprinid herpesvirus 2. *Aquat Anim Health*. 2016;28(3):150-160.
7. Alexander AB, Parkinson LA, Grant KR, Carlson E, Campbell TW. The hemic response of white-spotted bamboo sharks (*Chiloscyllium plagiosum*) with inflammatory disease. *Zoo Biol*. 2016;35(3):251-259.
8. LePage V, Young J, Dutton CJ, et al. Diseases of captive yellow seahorse *Hippocampus kuda* Bleeker, pot-bellied seahorse *Hippocampus abdominalis* lesson and weedy seadragon *Phyllopteryx taeniolatus* (Lacépède). *J Fish Dis*. 2015;38(5):439-450.
9. Anderson P, Berzins I, Fogarty F, Hamlin H, Guillette L. Sound, stress, and seahorses: the consequences of a noisy environment to animal health. *Aquaculture*. 2011;311(1–4):129-138.

10. Skalkos ZMG, Van Dyke JU, Whittington CM. Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus abdominalis*. *J Comp Physiol B*. 2020;190(5):547-556.
11. Campbell TW, Appendix A. Stains and solutions used in hematology and cytology. In: Campbell TW, ed. *Exotic Animal Hematology and Cytology*. 4th ed. Wiley Blackwell; 2015:377-381.
12. Campbell TW, Appendix B. Hematologic values. In: Campbell TW, ed. *Exotic Animal Hematology and Cytology*. 4th ed. Wiley Blackwell; 2015:383-391.
13. Friedrichs KR. ASVCP reference interval guidelines: determination of *de novo* reference intervals in veterinary species and other related topics. *Vet Clin Pathol*. 2012;41(4):441-453.
14. Le Boedec K. Sensitivity and specificity of normality tests and consequences on reference interval accuracy at small sample size: a computer-simulation study. *Vet Clin Pathol*. 2016;45(4):648-656.
15. Grant KR. Fish hematology and associated disorders. *Vet Clinics North Am: Exotic Animal Pract*. 2015;18:83-103.
16. Eshar D, Ammersbach M, Shacham B, Katzir G, Beaufrère H. Venous blood gases, plasma biochemistry, and hematology of wild-caught common chameleons (*Chamaeleo chamaeleon*). *Can J Vet Res*. 2018;86:106-114.
17. Tavares-Dias M, de Moraes FR. Leukocyte and thrombocyte reference values for channel catfish (*Ictalurus punctatus* Raf), with an assessment of morphologic, cytochemical, and ultrastructural features. *Vet Clin Pathol*. 2007;36(1):49-54.
18. Alleman AR, Jacobson ER, Raskin RE. Morphologic, cytochemical staining, and ultrastructural characteristics of blood cells from eastern diamondback rattlesnakes (*Crotalus adamanteus*). *Am J Vet Res*. 1999;60(4):507-514.
19. Campbell TW. Chapter 5: peripheral blood of fish. In: Campbell TW, ed. *Exotic Animal Hematology and Cytology*. 4th ed. Wiley Blackwell; 2015:97-114.
20. Arnold JE. Hematology of the sandbar shark, *Carcharhinus plumbeus*: standardization of complete blood count techniques for elasmobranchs. *Vet Clin Pathol*. 2005;34(2):115-123.

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