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Blood recovery of wild Mekong snail-eating turtles (*Malayemys subtrijuga* Schlegel and Müller, 1845) in captivity from leech infestation

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ABSTRACT

Blood cell counts are valuable diagnostic tools for assessing the health status of chelonians, however, reference standards for healthy blood parameters in various turtle species are lacking. In this study, forty wild female *Malayemys subtrijuga* were captured from ponds in Kasetsart University, and transported to laboratory for recuperating in captivity. All turtles were infected with a single leech species, *Placobdelloides siamensis*, with a mean of 513.7 ± 164.9 individuals per turtle, and exhibited penetrating and lesion wounds from leech infestations on both their skin and shell. Subsequently, they were cleaned and treated to eliminate ecto- and endoparasites before the recuperation period began. The turtles did not exhibit significantly differences in weight, carapace length (CL), red blood cell count (RCC), and white blood cell count (WCC) with a mean of 654.2 ± 199.9 g, 15.0 ± 2.5 cm, 327,080 ± 70,156 cells/mm^3, and 73,340 ± 15,859 cells/mm^3, respectively, during the initial records (week 0). However, after being maintained for 17 weeks, their health significantly improved in term of their blood parameters (RCC and WCC) and weight, except CL which remained unchanged, with a mean of 491,470 ± 16,169 cells/mm^3, 18,790 ± 1496 cells/mm^3, and 738.9 ± 191.5 g, respectively. Therefore, the health status obtained in this study can be used as a reference for blood parameters, weight, and recuperation period for the treatment of ill wild *M. subtrijuga* in captivity or as part of conservation management programs for turtles.

1. Introduction

Leech infestation is a common occurrence among various aquatic and semiaquatic organisms, especially vertebrate animals, which can lead to anemia and hemoparasite infection through lesions (Saumure and Beane 2001; de Campos Brites and Rantin 2004; Siddall et al., 2005; Moser et al., 2006; Mihalca et al., 2004; Readel et al., 2008; López-Jiménez and Oceguera-Figueroa 2009; Tiberti and Gentilli 2010; Chiangkul et al., 2018). The Siamese shield leech (*Placobdelloides siamensis* (Oka, 1917)) is the most widely distributed glossiphoniid leech among various turtle species in Thailand, including the Mekong snail-eating turtle (*Malayemys subtrijuga* Schlegel and Müller, 1845) (Brophy 2004; Chiangkul et al., 2018; Trivalairat et al., 2020). Despite its parasitic role on turtles in the ecosystem, the effect of this sanguivore on hosts is poorly understood in Thailand. Studies on the effects of leech infestation on wild turtles, such as Mekong snail-eating turtles, are necessary to understand the impact of leeches on the health of wild turtle populations.

Hematological analysis is an important diagnostic tool for evaluating the health status of vertebrate animals (Franzmann 1985). Changes in hematologic activities, such as the chemistry, morphology, or hemocyte count, are associated with certain health issues and diseases (Work et al., 1998; Aguire and Balazs 2000; Swimmer 2000). However, the different hematologic activities are also influenced by various factors like size, gender, age, nutrients, habitats, or seasons, both intraspecific and interspecific species (Whiting et al., 2007; Mattee 2014). In some reptile

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populations, including *Malayemys* turtles, these parameters have limited diagnostic value due to the lack of basic baseline parameters (Duguy 1970; Frair 1977). These turtles are widely distributed across Thailand and play an important role in biological control for water snails, especially invasive species that destroy vegetation products, and golden apple snails (*Pomacea canaliculata* Lamarck, 1819), which are also widely distributed in Thailand (Carlsson et al., 2004). However, the natural parasite *P. siamensis* of this turtle species impairs the host, with some individuals dying from blood loss caused by the leech (Chiangkul et al., 2018; Trivalairat et al., 2020). Hence, *Malayemys* turtles, particularly *M. subtrijuga*, were selected for this study to provide hematologic reference information for turtles in Thailand.

The aim of this study was to investigate the recovery of wild *M. subtrijuga* in a captive environment to establish a reference for their normal health status and recovery time from leech infestations, which can be fatal.

2. Materials and methods

2.1. Capture and identification techniques

Forty wild Mekong snail-eating turtles (*Malayemys subtrijuga*) were captured at Kasetsart University (13°50′53.6"N, 100°33′47.3"E) during the night while they were sleeping on the pond bank to avoid harm. Each individual was identified by their narrow nasal strips, which numbered at least six (*M. macrocephala* (Gray, 1859) has four or fewer wide nasal strips) (Das 2010). The turtles were also sexed by their tail characters, with males having longer tails and females having shorter tails, and using the probe technique on their cloaca (Keithmaleesatti 2008).

2.2. Population investigation

Malayemys subtrijuga were employed with a mark-recapture technique to estimate the population size (Southwood and Henderson 2000). They were closely monitored during the study period to ensure accurate estimation of the population size.

$$\text{Population size estimate} = \frac{\text{Marked sample released x Size of second sample}}{\text{Recaptured marked sample}}$$

2.3. Turtle preparation

On 2 November 2018, a total of forty mature female Mekong snail-eating turtles (*M. subtrijuga*) were collected from Kasetsart University ponds using hand technique during nighttime (08:00–10:00 p.m.). No males were found during the survey, and the turtles were stored in a plastic container ($64 \times 31 \times 48$ cm^3) with water from the captured site. The container was transported to the laboratory in Department of Zoology, Faculty of Science, Kasetsart University for further examination. The turtles' weight (g) and carapace length (CL) (cm) were measured, and leeches on the outer part of the turtles were removed using forceps. All the collected leeches were placed in a plastic container measuring $30 \times 20 \times 20$ cm^3, filled with water up to three-fourths of its capacity, and equipped with an oxygen pump. Subsequently, these leeches underwent identification and were deposited at the Zoological Museum within the Department of Zoology, Faculty of Science, Kasetsart University, without assigned deposit numbers. To eliminate endoparasites, the turtles were treated with metronidazole (25 mg/kg) to eliminate bacteria and protozoa and albendazole (50 mg/kg) to deworm them (Klingenberg 1993; Knotkova et al., 2005; Gibbons 2014). The outer parts of the turtles were cleaned with concentrated salt-dissolved water to eliminate ectoparasites.

2.4. Treatments

Each cleaned turtle was maintained at room temperature in a plastic container ($57 \times 32 \times 33$ cm^3) filled with water to one-third of the total volume and fitted with an oxygen pump, with pieces of pottery higher than the water level for basking, and the animals were fed Mazuri® Aquatic Turtle Diet (PMI Nutrition International LLC, a subsidiary of Land O'Lakes, Inc.) once a day. All containers were placed approaching slight sunlight, and the water was changed every day. They were maintained in the laboratory for treatment and recuperation for 17 weeks (week 0–17). Afterwards, all individuals were released at the collection site.

2.5. Blood collection

Approximately 6% of the turtle's body weight was collected as blood volume, with a maximum of 0.6 ml per 100 g body weight but not exceeding 2 ml per collection (Beaupre et al., 2004; Parasuraman et al., 2013). Once a suitable vessel was identified, the skin over the puncture site was cleaned with 70% ethanol, and a few drops of povidone-iodine were applied after collecting the blood to disinfect the area. Blood was collected from the caudal vein of each turtle using a plastic syringe every week (intervals of 7 days), and the body weight was recorded starting from the initial collection date (week 0) until the end of the experiments (week 17). The collected blood was immediately transferred into heparinized Vacutainer® tubes and stored at −20 °C. The blood samples were later used to analyze the red blood cells (RBC) and white blood cells (WBC) using the techniques described by Senthikumar Naidu (2011).

2.6. Red blood cell count (RCC)

Approximately 0.5 ml of blood was mixed with 100 ml of Hayem's fluid at a 1:200 dilution to maintain isotonic conditions for 2 min. Hayem's fluid comprises 0.5 g of sodium chloride (NaCl), 2.5 g of sodium sulfate (Na_2SO_4), 0.25 g of mercuric chloride ($HgCl_2$), and 100 ml of distilled water (Senthikumar Naidu 2011). A few drops were then discarded from the pipette on the hemocytometer, and the hemocytometer was slightly tilted with a small volume of fluid to introduce under the cover slip, which was placed on the counting chamber. After washing for a few minutes, the counting chamber was placed on the stage of the light microscope to count the RBC. Blood cells were counted in four corner squares plus the central square. The area of each corner square and the central square was 1 mm × 1 mm, and the depth of each square was 0.1 mm.

$$\text{Total red or white blood cell counts (RCC or WCC)} \left(\text{cells} / \text{mm}^3\right) = \frac{(\text{Number of RCC or WCC x Dilution ratio x Depth (mm)})}{\text{Number of chambers counted}}$$

2.7. White blood cell count (WCC)

Approximately 0.5 ml of blood or adapted blood volume was diluted with 10 ml of Tuck's fluid for a 1:20 dilution for 1 min. Tuck's fluid consisted of 1.5 ml of 3% acetic acid (lysed RBC) and 1 ml of 1% gentian

Table 1
Normality test of model variables in *Malayemys subtrijuga* (n = 40 individuals/week) based on Shapiro-Wilk test: red blood cell count (RCC, cells/mm^3), white blood cell count (WCC, cells/mm^3), weight (g), carapace length (CL), and number of leeches (individuals)./= normal distribution; × non-normal distribution. Parameter (week).

Parameters	Shapiro-Wilk test	*p*-values (*p*)	Normal distribution
CL (0)	0.00564	p < 0.05	×
Weight (0)	0.00058	p < 0.05	×
Weight (17)	0.00011	p < 0.05	×
RCC (0)	0.00082	p < 0.05	×
WCC (0)	0.00111	p < 0.05	×
Leech (0)	0.05377	p > 0.05	/

*p > 0.05; normal distribution.

violet for staining nuclei of white blood cells to increase visibility and 100 ml distilled water (Senthikumar Naidu 2011). Then, a few drops were discarded on the hemocytometer and placed under a microscope to count the WBC.

2.8. Statistical analyses

A linear mixed model was employed to analyze the changes in red blood cell and white blood cell counts (RCC and WCC), as well as weight, and compared to the results obtained during week 0. The normality of RCC, WCC, weight, CL, and the number of leeches during week 0, along with weight during week 17, was assessed using the Shapiro-Wilk test. Results indicated that the number of leeches was normally distributed ($p > 0.05$), while the others were non-normal (Table 1). Spearman correlation was used to investigate the relationship between the number of leeches, RCC, WCC, carapace length, and weight. All statistical analyses were performed using the R programming language.

3. Results

3.1. Population estimation

The first capturing event on 18 December 2016 revealed a total of 71 individuals of Mekong snail-eating turtle (*Malayemys subtrijuga*) (54 females and 17 males). The turtles were marked by cutting a small notch on the posterior marginal scute before being released at the capture sites. After two weeks (2 January 2017), the second capturing event revealed a total of 60 individuals (22 females and 38 males), of which 21 individuals were recaptured (8 females and 13 males). Estimations using the mark-recapture technique demonstrated that the total population of *M. subtrijuga* was 202.9 individuals, with 148.5 individuals being female and 49.7 individuals being male. Therefore, female *M. subtrijuga* individuals from the larger population were chosen as subjects for this study.

3.2. Host specimens

Forty individuals of the wild female *M. subtrijuga* were collected on 2 November 2018 from ponds at Kasetsart University. Each individual was assigned a number according to the sequence in which it was collected (no. 1–40). The turtles had a mean carapace length (CL) of 15.0 ± 2.5 cm (range 11.2–18.9 cm) and weight of 654.2 ± 199.9 g (range 408.76–1011.71 g) (Table 2). Significant differences were observed in CL ($p = 0.006$) and weight ($p = 0.001$) among individuals. There was no change in CL during the recuperation period. CL was significantly and

Table 3
Spearman's correlation of model variables in *Malayemys subtrijuga* (n = 40 individuals/week): red blood cell count (RCC, cells/mm^3), white blood cell count (WCC, cells/mm^3), weight (g), carapace length (CL), and number of leeches (individuals). Parameter (week).

Parameters	*p*-values (*p*)	Rho (*r*)
RCC (0 × 17)	1.191e-14	0.892*
RCC (0) × WCC (0)	7.007e-7	−0.694*
RCC (17) × WCC (17)	<2.200e-16	1.000*
RCC (0) × Weight (0)	5.859e-5	−0.591*
RCC (17) × Weight (17)	9.945e-7	−0.687*
WCC (0 × 17)	4.274e-5	−0.600*
WCC (0) × Weight (0)	0.510	0.107
WCC (17) × Weight (17)	9.945e-7	−0.687*
Weight (0 × 17)	<2.2e-16	0.986*
Weight (0) × CL (0)	<2.2e-16	0.949*
Weight (17) × CL (17)	<2.2e-16	0.953*
Leech (0) × CL (0)	0.685	−0.079
Leech (0) × Weight (0)	0.7717	−0.121
Leech (0) × WCC (0)	0.5075	−0.003
Leech (0) × RCC (0)	0.5075	−0.090
Leech (0) × RCC (0)	0.2898	−0.090

*p < 0.05; significant statistical difference.

Table 2
Means and ranges of blood cell counts (red blood cell count (RCC) and white blood cell count (WCC)) (cells/mm^3) and weight of *Malayemys subtrijuga* in each week (n = 40 individuals/week) throughout the experimental period (week 0–17).

Weeks	RCC (cells/mm^3)		WCC (cells/mm^3)		Weight (g)	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Week 0 (2 Nov 18)	327,080 ± 70,156	230,000–438,000	73,340 ± 15,859	51,200–97,350	654.2 ± 199.9	408.8–1011.7
Week 1 (9 Nov 18)	308,600 ± 73,832*	205,500–424,750	71,870 ± 13,901	50,400–88,200	605.9 ± 184.9*	366.6–904.1
Week 2 (16 Nov 18)	324,230 ± 70,135	230,000–434,500	67,760 ± 13,680*	48,200–86,800	576.7 ± 195.0*	257.2–907.9
Week 3 (23 Nov 18)	338,250 ± 64,973*	248,250–441,500	58,920 ± 8700*	45,000–68,400	650.9 ± 197.1	411.6–970.9
Week 4 (30 Nov 18)	348,300 ± 64,638*	250,250–448,000	53,180 ± 4156*	47,050–59,200	674.5 ± 192.9*	446.2–983.9
Week 5 (7 Dec 18)	359,370 ± 54,547*	289,000–452,350	44,397 ± 2494*	41,700–48,300	672.0 ± 198.1*	425.0–988.2
Week 6 (14 Dec 18)	379,350 ± 51,012*	317,500–453,250	42,949 ± 2053*	40,700–46,500	676.2 ± 193.3*	448.1–987.0
Week 7 (21 Dec 18)	388,900 ± 49,056*	343,250–458,750	40,950 ± 1635*	39,250–43,900	694.3 ± 206.2*	448.7–989.5
Week 8 (28 Dec)	399,310 ± 48,856*	351,000–469,450	39,110 ± 1667*	37,100–42,000	694.7 ± 205.9*	449.7–988.2
Week 9 (4 Jan 19)	408,380 ± 44,421*	363,400–469,250	36,465 ± 2234*	33,800–40,750	696.4 ± 204.5*	453.7–989.1
Week 10 (11 Jan 19)	415,390 ± 45,841*	368,500–471,250	29,390 ± 998*	27,550–30,400	700.0 ± 205.0*	457.0–994.7
Week 11 (18 Jan 19)	425520 ± 42,560*	379,850–480,500	28,660 ± 1431*	27,100–30,850	704.4 ± 205.0*	460.3–998.6
Week 12 (25 Jan 19)	433,340 ± 39,937*	394,450–394,450	27,240 ± 2079*	24,450–30,600	708.6 ± 204.3*	467.9–1000.9
Week 13 (1 Feb 19)	442,750 ± 34,521*	406,750–489,250	24,250 ± 3972*	18,750–30,900	713.0 ± 203.4*	472.3–1004.6
Week 14 (8 Feb 19)	451,300 ± 33,939*	412,500–493,500	23,430 ± 2558*	20,300–27,750	714.1 ± 201.9*	473.2–1002.9
Week 15 (15 Feb 19)	473,580 ± 24,734*	438,500–507,450	21,000 ± 2230*	17,750–17,750	732.0 ± 193.5*	486.9–1008.4
Week 16 (22 Feb 19)	483,890 ± 23,299*	447,000–514,450	20,800 ± 1918*	17,550–22,500	734.1 ± 192.9*	490.2–1009.1
Week 17 (1 Mar 19)	491,470 ± 16,169*	467,000–514,500	18,790 ± 1496*	17,050–20,500	738.9 ± 191.5*	497.3–1012.3
All weeks (n = 720)	399,945 ± 74,444	205,500–514,500	40,138 ± 18,786	17,050–97,350	685.6 ± 200.7	257.2–1012.3

*p < 0.05; significant statistical difference compares with week 0.

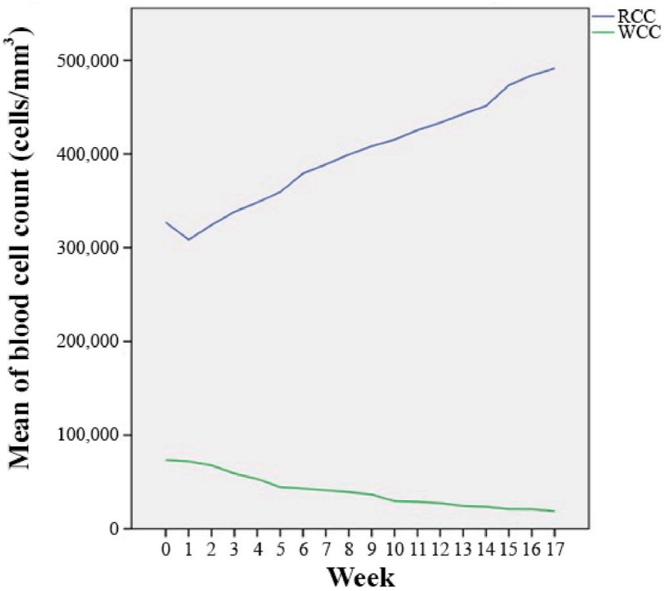

Fig. 1. Analysis of the mean red blood cell count (RCC) (blue line) and white blood cell count (WCC) (green line) of *Malayemys subtrijuga* during captivity recovery from 2 November 2018 (week 0) to 1 March 2019 (week 17). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

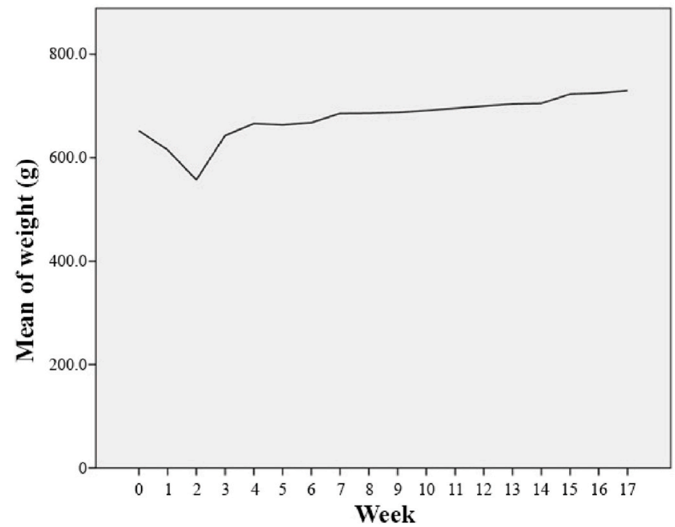

Fig. 2. Analysis of the mean weight of *Malayemys subtrijuga* during captivity recovery from 2 November 2018 (week 0) to 1 March 2019 (week 17).

positively correlated with weight ($r_{\text{weight (0)}} = 0.949$, $r_{\text{weight (17)}} = 0.953$) (Table 3). These findings indicate a relatively homogenous population with no significant variations in CL and weight. The positive correlation between CL and weight suggests that larger individuals tend to be heavier.

Furthermore, Siamese shield leeches (*Placobdelloides siamensis*) were extracted from all collected turtles, and each host individuals had a prevalence of 100%, with a mean intensity of 513.7 ± 164.9 individuals/turtle (range 254–794 individuals/turtle). The number of leeches at week 0 was found to have negligible correlation with all parameters, including RCC ($r = -0.090$), WCC ($r = -0.003$), weight ($r = -0.121$), and CL ($r = -0.079$) (Table 3).

3.3. Initiation phase (week 0–3)

On the first day of the recuperation (2 November 2018, week 0), all *M. subtrijuga* specimens demonstrated a mean red blood cell count (RCC) of 327,080 ± 70,156 cells/mm^3 (range 230,000–438,000 cells/mm^3) and white blood cell count (WCC) of 73,340 ± 15,859 cells/mm^3 (range 51,200–97,350 cells/mm^3), as shown in Table 2. There were no significant differences in RCC ($p = 0.617$), WCC ($p = 0.950$), or weight ($p = 0.150$) between individuals (Table 3). Furthermore, the results from week 0 demonstrated a negative correlation between RCC and WCC ($r = -0.694$) as well as weight ($r = -0.591$).

After eliminating parasites and treating the turtles in the laboratory for a week (9 November 2018, week 1), decreases in weight, RCC, and WCC were observed, with means of 605.9 ± 184.9 g (range 366.6–904.1 g), 308,600 ± 73,832 cells/mm^3 (range 205,500–424,750 cells/mm^3), and 71,870 ± 13,901 cells/mm^3 (range 50,400–88,200 cells/mm^3), respectively (Table 2) (Figs. 1 and 2). Subsequently, in week 2 (16 November 2018), the RCC showed a trend towards increase to a mean of 324,230 ± 70,135 cells/mm^3 (range 230,000–434,500 cells/mm^3), followed by weight which showed a trend towards increase to a mean of 650.9 ± 197.1 g (range 411.6–970.9 g) in week 3, respectively, and continued to increase up to week 17. In contrast, the WCC continued to decrease from week 0 to week 17.

3.4. Recovery phase (week 3–17)

After being maintained continuously for 17 weeks, the mean RCC and weight of *M. subtrijuga* increased to 491,470 ± 16,169 cells/mm^3 (range 467,000–514,500 cells/mm^3) and 738.9 ± 191.5 g (range 497.3–1012.3 g), respectively, in week 17 (1 March 2019), which represents a 150.3% and 113.0% increase compared to the first captured date (2 November 2018, week 0). There was a positive correlation between the RCC and weight and the first captured date ($r_{\text{RCC(0x17)}} = 0.892$, $r_{\text{weight(0x17)}} = 0.986$), while the WCC decreased to 18,790 ± 1496 cells/mm^3 (range 17,050–20,500 cells/mm^3), which represents a 25.6% decrease compared to the first captured date, and there was a negative correlation ($r_{\text{WCC(0x17)}} = -0.600$).

These findings suggest that the RCC and weight of *M. subtrijuga* trended to increase continuously along the recuperation process, in contrast to the WCC, which continuously decreased since week 0. Moreover, in week 17, the correlation coefficient of RCC and WCC increased significantly ($r = 1.000$), as well as weight and CL ($r = 0.953$), while the correlation between RCC and weight, and WCC and weight slightly decreased significantly ($r = -0.687$). Furthermore, length and width of red blood cell size from these turtles were measured (25 cells/individuals) and exhibited with a mean of 14.77 ± 1.43 μm (range 10.60–19.20 μm) and 9.84 ± 1.02 μm (range 6.50–14.50 μm), respectively. The ratio between length and width was 1.51.

Overall, these results suggest that the RCC and WCC of female *M. subtrijuga* that have eliminated parasites and been treated in a proper captive environment for at least 17 weeks can be estimated using linear formulas: RCC trend ($y_{RCC} = 2.99E5 + 1.06E4*x$: y_{RCC} = red blood cell count (cells/mm^3), x = time (weeks)); and WCC trend ($y_{WCC} = 7.13E4 + -3.28E3*x$: y_{WCC} = white blood cell count (cells/mm^3), x = time (weeks)) (Fig. 3).

3.5. Leech effects

Leeches primarily colonized the outer parts of the collected *M. subtrijuga*, such as the carapace, plastron, head, axilla, groin, and cloaca (no. 7 and 8) (Fig. 4A–B). Some were also found accidently in the

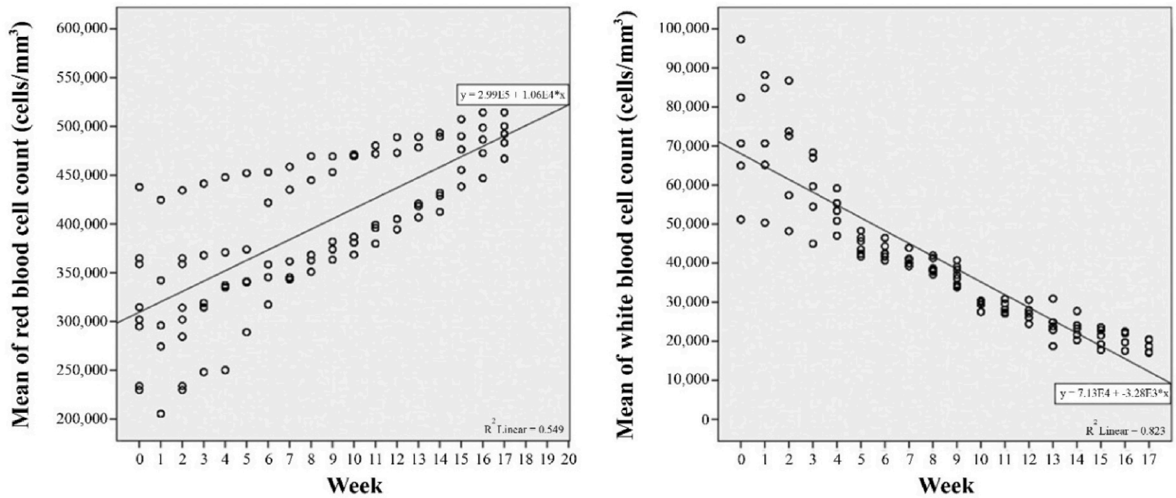

Fig. 3. Trend analysis of red blood cell count (RCC) (left) and white blood cell count (WCC) (right) of *Malayemys subtrijuga* during captivity recovery from 2 November 2018 (week 0) to 1 March 2019 (week 17). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

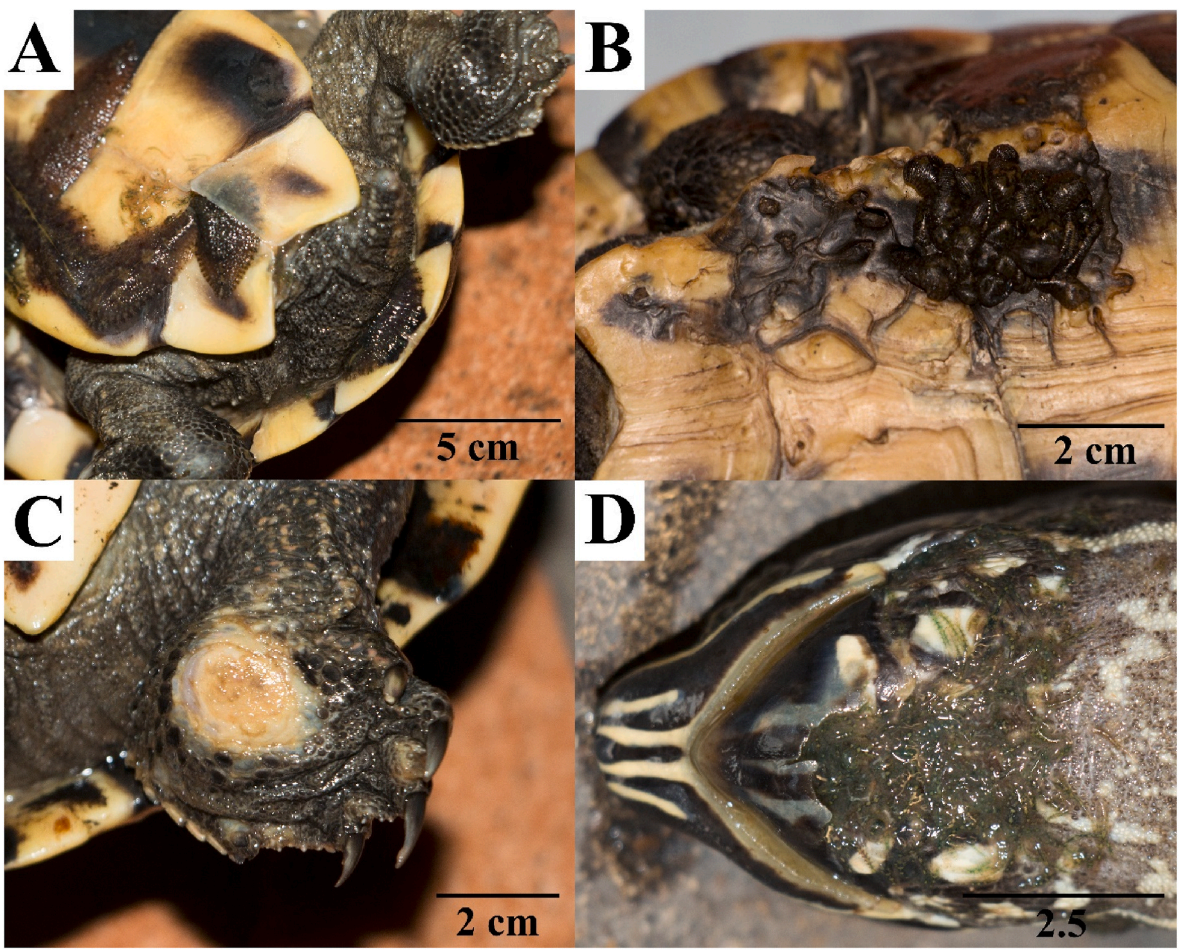

Fig. 4. Symptoms of *Placobdelloides siamensis* infection on *Malayemys subtrijuga*: (A) Leech penetration beneath the keratin layer (scute) on plastron from no. 8; (B) Shell holes resulting from leech penetration on plastron from no. 7; (C) Epidermal lesion on the hind foot from no. 6; (D) Keratin mandible jaw with leech consumption from no. 4.

buccal cavity (no. 8). The scales on the infected limbs and feet usually had shade on the epidermal layer, and the dermal layer was usually penetrated for consumption (no. 6), especially the hardest part of the keratin mandible jaw (no. 4) (Fig. 4C–D). The keratin layer scutes on the plastron were also shaded, and the bone tissues were penetrated through the soft tissue layer, then colonized for egg laying. Occasionally, leeches deposited and raised their eggs on the carapace surfaces, with approximately 321.0 ± 65.6 eggs per clutch (range 214–426). Moreover, most

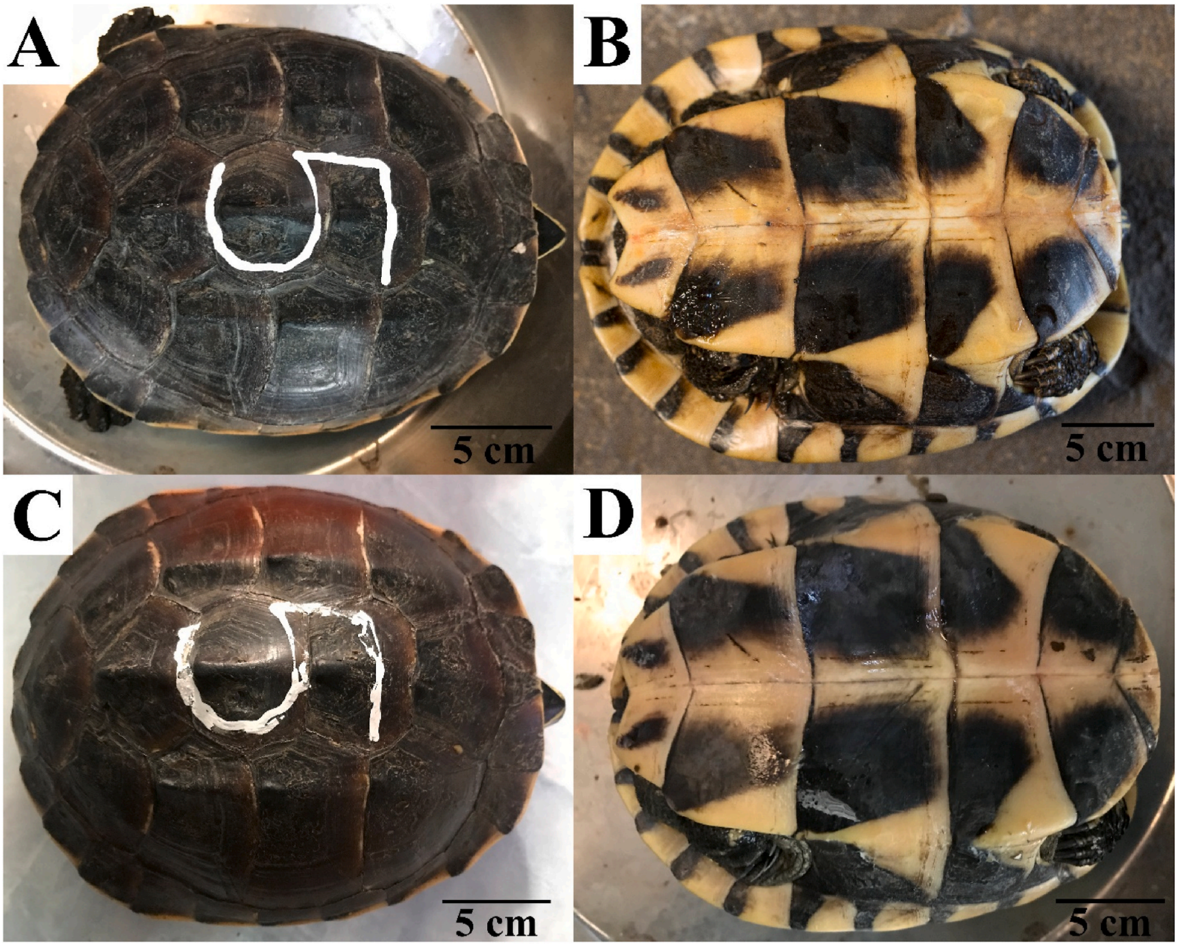

Fig. 5. Morphological comparison of *Malayemys subtrijuga* no. 5 between week 0 (A and B) and week 17 (C and D).

collected turtles showed low activity, anorexia, and weight loss in the first few weeks of recuperation (week 0–1) before gradually recovering their health as seen in Fig. 5 (no. 5).

4. Discussion

4.1. Blood profiles for a wild population

This study is the first to report on the use of red blood cell count (RCC) and white blood cell count (WCC) as diagnostic tools for the health of *Malayemys subtrijuga*. The study found that wild individuals infected with *Placobdelloides siamensi* had an average RCC of 325,812.5 ± 77,187.6 cells/mm^3 and an average WCC of 73,324.4 ± 17,838.6 cells/mm3. These values were similar to those of other wild Geoemydidae turtles, such as *Hieremys annandalii* (Boulenger, 1903) from Bangkok, Thailand (total RCC 275,000 ± 94,000 cells/mm^3 (male 293,000 ± 109,000 cells/mm^3, and female 258,000 ± 77,000 cells/mm^3), and total WCC 11,660 ± 6590 cells/mm^3 (male 13,620 ± 7610 cells/mm^3, and female 9700 ± 4810 cells/mm^3) and *Mauremys leprosa* (Schweigger, 1812) from Almeria, Spain (total RCC 401,310 ± 11,0470 cell/mm^3, and 540,280 ± 20,330 cell/mm^3 in summer and autumn, respectively), with a similar body weight (349.7 ± 84.1 g for summer and 319.4 ± 17.5 g for autumn, respectively) (Pagés et al., 1992; Chansue et al., 2011). However, the RCC and WCC values were higher than those of *Ma. rivulata* from Canakkale, Turkey (Tosunoglu et al., 2011). Unfortunately, the study did not include measurements of pack red blood cell (PCV) count, which provides the volume percentage of red blood cells in the blood. Additionally, a few studies on the PCV of *M. subtrijuga* have reported a mean of 22.3 ± 1.4 cm3/100 cm3 (range 18–32 cm3/100 cm3) (Frair 1977).

In summary, blood parameters often vary due to various factors, including intra- and interspecific relationships, such as species, sex, age, physiological state, season, temperature, habitat, and nutrition, especially sanguivore parasites like leeches (Pagés et al., 1992; Samour et al., 1998; Wang et al., 1999; Dickinson et al., 2002; Peterson 2002; Gicking et al., 2004; Keller et al., 2004; Campbell 2006; Metin et al., 2006, 2008; Tavares-Dias et al., 2009; Akbulut et al., 2010). Therefore, the intervals of blood parameters could be used as diagnostic tools for determining and assessing the health of wild *M. subtrijuga* turtles with a range of carapace lengths and weights of 13–15 cm and 350–400 g, respectively, and provides reference values for RCC and WCC. The study also highlights the importance of considering various factors that can affect blood parameters when interpreting their values.

4.2. Symptoms of infection

The colonization pattern of leeches was predominantly observed on the external part of *M. subtrijuga*, including the carapace, plastron, head, axilla, groin, and cloaca, with occasional occurrences in the buccal cavity. Leech infection caused physical damage to the host's shell and epidermal tissues, resulting in lesion wounds and shell deformation. The scales on the infected limbs and feet exhibited shading on the epidermal layers, and the dermal layer was usually penetrated for consumption, particularly the hardest part of the keratin mandible jaw. The keratin layer scutes on the plastron were also shaded, and the bone tissues were penetrated through the soft tissue layer for colonization and egg-laying. In some cases, leeches deposited and raised their eggs on the carapace surface (Chiangkul et al., 2018; Trivalairat et al., 2020).

The jawless leech, which belongs to the Rhynchobdellida order, feeds by utilizing its muscular proboscis, which features micropores at its tip where proteolytic enzymes are released to aid in the penetration of the host's epidermal tissues and shell (Sawyer 1986; Moser et al., 2009; Chiangkul et al., 2021a). This allows the leech to suck blood from the capillaries and consume soft tissues, keratinous scales, and bone tissues by piercing the hard parts with its needle-liked proboscis, much like an ice pick (Sawyer 1986; Kutschera 1992; Chiangkul et al., 2021a). This feeding strategy differs from that of the Arhynchobdellida order of leeches, which possess jaw (Mann 1962). The consumption of hard parts such as shells and keratinous jaws leads to permanent porosity or deformation that cannot be regenerated.

In this study, *P. siamensis* infection in each individual resulted in anemia, caused by a significant decrease in red blood cells and an immune response that increased white blood cells. Similarly, leech-infected Geoffroy's side-necked turtle (*Phrynops geoffroanus*) from Uberabinha river that also showed acute normocytic anemia (de Campos Brites and Rantin 2004). Turtles with anemia may experience reduced activity level due to decreased oxygen-carrying capacity, leading to fatigue (Saggese 2009). Additionally, there was a significant difference in weight between week 0 and week 17, including anorexia in wild-caught turtles, which may be due to malnutrition.

Therefore, the prevalence and intensity of *P. siamensis* in the host population provide important information regarding the potential impact of these parasites on the health of turtles. The leech load can cause wound lesions, shell deformation, anorexia, malnutrition, fatigue, and severe anemia, ultimately lead to the degradation of health status or death. In nature, turtles control their leech load by basking in sunlight during the day or in the air at night (Ernst 1971; McAuliffe 1977; Koffler et al., 1978). *P. siamensis* is one of the factors that reduces the fitness of *M. subtrijuga* in freshwater ecosystems of Thailand.

To mitigate the spread of *P. siamensis* infection, it is essential to consider restricting turtle releases, particularly the traditional practice of "making merit," which is not native to these areas. Within Thai Buddhist tradition, "making merit" involves the release of captive animals, such as turtles, fish, birds, or snails, into their natural habitats. This compassionate act signifies kindness toward all living beings and is believed to contribute to the accumulation of positive spiritual karma. The practice of "making merit" poses a challenge in the context of conservation in Thailand, as it conflicts with scientific efforts. Many Thai people firmly believe in the accumulation of good karma and frequently release animals, even in areas where laws and supporting evidence indicate potential harm to local species or improper habitat, leading to adverse consequences. Therefore, if prohibiting people from practicing "making merit" is not feasible, it becomes imperative, especially in conservation scenarios, to ensure that released turtles are free of parasites and undergo recuperation before returning to the wild.

4.3. Treatments

Most wild turtles, including *M. subtrijuga*, are susceptible to parasite-host relationships. Parasites, both ecto- and endoparasites, can have a direct impact on the blood health, nutrition, and immune response of the hosts. Examples of parasites found in turtles include leeches, such as *P. siamensis*, which were present in all individuals in this study, and ticks on the outer parts, as well as blood flukes (*Platt sinuosus* Roberts and Bullard, 2018 and *Pl. snyderi* (Platt and Sharma, 2012)) and *Haemogregarina pellegrini* Laveran and Pettit (1910) in the blood system (Ray and Bhattacharjee 1984; Frye 1991; Mader 1996; Siddall and Desser 2001; Kikuchi and Fukatsu 2002; Knotkova et al., 2005; Stacy et al., 2011; Dvorakova et al., 2015; Chiangkul et al., 2018, 2021b; Javanbakht and Sharifi 2014; Úngari et al., 2018; Bullard et al., 2019; Trivalairat et al., 2020). The elimination of parasites and recuperation in a proper captive site represent treatment methods and can be used to evaluate the health status of turtles.

In this study, concentrated salt-dissolved water was used to eliminate freshwater leeches. Freshwater leeches are very sensitive to salinity, and salt is also a worldwide anti-leech substance used for the removal or killing of leeches (Harley et al., 2013; Bam et al., 2015; Joslin et al., 2017). For the endoparasites of *M. subtrijuga*, Metronidazole and Albendazole were used. Metronidazole is a common drug used for treating bacterial and protozoal infections in various turtles, such as *Staphylococcus* spp. in red-eared slider turtle (*Trachemys scripta elegans* (Wied-Neuwied, 1839)) which can cause systemic coagulation depletion of clotting factors, followed by hemorrhages, as well as *Hemogregarine* spp. (haemoparasites) which can cause the rupture of red blood cells (Laveran and Pettit 1910; McAuliffe 1977; Knotkova et al., 2005; Angus and van der Poll 2013; Dvorakova et al., 2015; Assan Kasim et al., 2017; Úngari et al., 2023). This drug also has a longer half-life in turtles (27 h) compared to mammals (Innis et al., 2006). Albendazole, on the other hand, is commonly used to eliminate helminths in mammals and reptiles, such as *Trichinella* spp. (Klingenberg 1993; Gibbons 2014; Jeong et al., 2015).

All of these procedures were carried out to minimize the impact of parasite-host associations that could potentially result in a decrease in red blood cells or an increase in white blood cells (immune responses) in *M. subtrijuga* before they were released into the wild or recuperated in captivity. Considering parasitic infections in turtles is critical since they can cause severe health issues and even death if left untreated (Hernandez-Divers and Schumacher 2009b). Appropriate management and therapy can assist in maintaining the health and welfare of captive turtles.

4.4. Implications for monitoring and management

Based on the presented data, it appears that the *M. subtrijuga* turtles recovered from their initial health issues and gradually increased in weight and RCC over time in captivity. However, during the first few weeks (week 1–3) after eliminating parasites and treating the turtles in the captivity, they showed little or no interest in the given diet until 14–17 days. This period coincides with the decrease in weight and RCC, which may have been influenced by changes in behavior and metabolism under captive conditions (Beery and Zucker 2011). In most animals, the amount of food intake is directly correlated with weight gain (Bhatnagar 2015). Similarly, these turtles exhibited an increase in weight only after showing an interest in their food. Nevertheless, the increase in RCC in week 2, before the turtles started eating again, suggested that it was not influenced by the amount of food intake. Therefore, the increase in RCC may have been due to the elimination of parasites, which limited factors that could potentially decrease red blood cells overtime (Owerkowicz et al., 2009). These results suggest that close monitoring and management of captive turtles during the recuperation period is important to ensure proper food intake and prevent any unexpected changes in blood parameters (Hernandez-Divers and Schumacher 2009a; Brown and Jacobson 2014).

After being held in captivity for 17 weeks, the turtles showed improvement in their blood health status and weight. Their RCC increased to 150.3% (491,470 ± 16,169 cells/mm^3), and their weight increased by 113.0% (739 ± 113.0 g), while their white blood cell count (WCC) decreased to 25.6% (18,790 ± 1496 cells/mm^3). The blood parameters of these recuperated turtles were better than that of the first capture at week 0 and were similar to those of healthy captive turtles in the same family (Geoemydidae). For instance, female *Cuora flavomarginata* in summer had an RCC of 490,000–1,200,000 cells/mm^3 and WCC of 800-10,420 cells/mm^3, *Kachuga smithii* had an RCC of 661,000 ± 58,000 cells/mm^3 (390,000–970,000 cells/mm^3), *M. caspica leprosa* had an RCC of 430,000 cells/mm^3 (319,000–548,000 cells/mm^3), *Ma. rivulata* with RCC 197,333 ± 40,252.56 cells/mm^3 (165,000–253,333 cells/mm^3) and WCC of 2479 ± 471.75 cells/mm^3 (1733–2866 cells/mm^3), and *Orlitia borneensis* Gray, 1873 had an RCC of 360,000 ± 200,000 cells/mm^3 and WCC of 7400 ± 3200 cells/mm^3 (Brown 1971; Frair 1977; Knotkova et al., 2005; Tosunoglu et al., 2011; Yang et al., 2014). However, there is no evidence regarding the recuperation time of the referred turtle species to estimate how long each turtle needs to be treated until healthy, except for *O. borneensis*, which were treated after suffering from shell necrosis and *haemogregarine* parasites (Knotkova et al., 2005). The infected *O. borneensis* showed the RCC and WCC at 260,000 ± 100,000 cells/mm^3 and 14,500 ± 6400 cells/mm^3 before increased to the normal ranges of healthy turtles after treatment for 24 months (Knotkova et al., 2005). However, the recuperated duration for each turtle may differ depending various factors such as species, size, gender, age, nutrients, habitats, or seasons (Whiting et al., 2007; Mattee 2014). In this study, even though the female *M. subtrijuga* turtles showed significant improvement in their blood parameters and weight after spending 17 weeks, their blood parameters were not within the normal range. The RCC and WCC trends continued to rise and decline, respectively, even towards the end of the study (week 17). The increase in weight and carapace length also suggests that the turtles were able to grow and develop normally during their recuperation. Therefore, these findings suggest that captive environments and proper treatment can promote the health of female *M. subtrijuga*, and that RCC and WCC can be useful indicators of their overall health. Furthermore, future research should explore the potential relationship between RCC and WCC to study the limit trend and normal range.

In conclusion, it is imperative to acknowledge that the utilization of captive environments for the rehabilitation and convalescence of sick or injured turtles is not bereft of challenges. For instance, stress and changes in behavior and metabolism could potentially impede the recovery and health of the animals (Beery and Zucker 2011). Therefore, it is crucial to diligently monitor the turtles during the recuperation phase to ensure appropriate food consumption and forestall any unforeseen alterations in blood parameters (Hernandez-Divers and Schumacher 2009a; Brown and Jacobson 2014). The careful management of turtles during their recovery phase could also aid in averting the transmission of infectious diseases and parasites that may affect other animals, particularly endangered or threatened species, or even the wild populations of the same species that may depend on captive breeding or conservation programs for their survival.

Ethical statement

This research was conducted in compliance with the ethical guidelines set forth by the Institute of Animals for Scientific Purpose Development (IAD) of Thailand and was approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Kasetsart University, under project number ACKU61-SCI-028.

CRediT authorship contribution statement

Poramad Trivalairat, Krittiya Chiangkul, Watchariya Purivirojkul: Conceptualization; Poramad Trivalairat, Krittiya Chiangkul, Watchariya Purivirojkul: Methodology; Poramad Trivalairat, Krittiya Chiangkul, Watchariya Purivirojkul: Investigation; Awirut Tassamakornd: Data curation; Poramad Trivalairat, Krittiya Chiangkul, Awirut Tassamakornd: Formal analysis; Poramad Trivalairat, Krittiya Chiangkul, Watchariya Purivirojkul: Supervision; Poramad Trivalairat, Krittiya Chiangkul: Writing - original draft; Poramad Trivalairat, Krittiya Chiangkul, Awirut Tassamakornd, Watchariya Purivirojkul: Writing - review & editing.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to Grammarly check and improve language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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