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## 2-PHENOXYETHANOL (2-PE) AND TRICAINA METHANESULFONATE (MS-222) IMMERSION ANESTHESIA OF AMERICAN HORSESHOE CRABS (*LIMULUS POLYPHEMUS*)

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**Abstract:** Despite extensive literature examining American horseshoe crab physiology, there are comparatively few publications addressing their medical care. Establishing anesthesia protocols for horseshoe crabs is integral to limiting the potential stress and pain associated with invasive procedures and for advancing euthanasia techniques. The objective of this study was to compare the effects of two immersion anesthetics, tricaine methanesulfonate (MS-222) at 1 g/L (buffered with sodium carbonate) and 2-phenoxyethanol (2-PE) at 2 mL/L, on horseshoe crabs. Twenty horseshoe crabs were assigned to one of two anesthetic treatment groups and individually anesthetized in natural seawater. Water quality, cardiac contractility, and hemolymph gas analytes were measured prior to anesthesia and at 30 min. Animals were monitored via heart rate, gilling rate, and sedation score every 5 min until recovered. Transcarapacial ultrasonography was used to obtain heart rate, gilling rate, and percent fractional shortening. Light or surgical anesthesia was produced in 10/10 animals in the 2-PE group and 8/10 animals in the MS-222 group. There was no significant difference in sedation scores, induction time (median 15 min), or recovery time (median 20.5 min). Gilling rate and cardiac contractility decreased during anesthesia, whereas heart rate did not. Hemolymph pH and pO<sub>2</sub> were not different among treatment groups or time points. Baseline pCO<sub>2</sub> was higher than pCO<sub>2</sub> at 30 min for both groups but significantly elevated only in the MS-222 group. This is attributed to increased activity during the handling of awake animals. Invasive blood pressure obtained via cardiac catheterization in two animals was markedly decreased during surgical anesthesia. In conclusion, 2-PE and MS-222 provided effective anesthesia with clinically useful induction and recovery times. 2-PE provided a subjectively more reliable and smoother anesthesia compared to MS-222.

**Key words:** American horseshoe crab, Atlantic horseshoe crab, blood pressure, hemolymph gas, immersion anesthesia, *Limulus polyphemus*.

### INTRODUCTION

The American horseshoe crab (HSC, *Limulus polyphemus*) is one of four extant HSC species. It is found in stable to declining numbers throughout the Gulf of Mexico and along the east coast of North America.<sup>27</sup> Despite its wide geographic range, the population is characterized as vulnerable, due primarily to unsustainable harvesting.<sup>27</sup> Current strategies to conserve this species include harvest regulations, habitat protection, and enhancing public awareness.<sup>26–28</sup> Displays incorporating HSCs are common in public aquaria, and as a result, this species is increasingly presented for veterinary care. Additionally, HSCs have for

decades been one of the most intensely studied aquatic invertebrates and continue to play a prominent role in biomedical research.<sup>5,9,14,16,20</sup>

HSCs have developed considerable physiologic adaptations for survival in a wide range of environments and to accommodate their unique life history, which can include periods of hypoxia greater than 24 hr during seasonal beach spawning.<sup>30</sup> The HSC is considered an osmoconformer and alters hemolymph osmolality to match that of its surroundings within a wide range of salinities.<sup>30</sup> It is highly tolerant of hypoxia and responds by decreasing blood pressure, heart rate (HR), and gilling rate (GR).<sup>23</sup> HSCs utilize hemocyanin as an extracellular oxygen-carrying molecule. In contrast to hemoglobin, hemocyanin holds only one oxygen molecule and exhibits a reverse Bohr effect characterized by increasing oxygen-hemocyanin affinity as pH decreases.<sup>4</sup> HSCs have a low-pressure open circulatory system driven by a single-chambered, elongated heart and possess several pairs of lamellate book gills on their ventrum for gas exchange.

Tricaine methanesulfonate (MS-222) and 2-phenoxyethanol (2-PE) are commonly used immersion anesthetics in aquatic animals. MS-222 is

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a benzocaine derivative that acts by blocking voltage-gated sodium channels. In fish, sedation with MS-222 results in dose-dependent cardiovascular and respiratory depression and is excreted primarily by the gills.<sup>17</sup> 2-PE has been used as a sedative, topical, and general anesthetic agent in fish and aquatic invertebrates.<sup>24</sup> The mechanism of action of 2-PE is unknown. Like MS-222, it causes depression of the cardiovascular and respiratory systems.<sup>15</sup>

Despite extensive literature examining the physiology of the American HSC, there are comparatively few publications addressing their medical care.<sup>1–3,10–13,18,19,29,32</sup> Specifically, controlled investigations outlining sedation and anesthesia are lacking. Establishing anesthesia protocols for HSCs is integral to limiting the potential stress and pain associated with diagnostic or surgical procedures and for advancing euthanasia techniques. The objective of this study was to compare the effects of two immersion anesthetics, MS-222 and 2-PE, on the quality of anesthesia and effect on physiologic variables in wild-caught American HSCs monitored via a sedation score, GR, HR, cardiac contractility, hemolymph gas analysis, and invasive blood pressure.

## MATERIALS AND METHODS

### Animals

Twenty-two wild-caught adult American HSCs (female  $n = 13$ , male  $n = 9$ ) were collected from nearby coastal waters in North Carolina, USA, under a scientific research collection permit (#1791359) from the North Carolina Division of Marine Fisheries. They were housed indoors divided into five tanks (volume 570–700 L), maintained on an open flow-through natural seawater system (flow rate 4–6 L/min), and provided with one or two air stones per tank. They were maintained on a 12-hr artificial light cycle, and windows provided additional natural light. All animals appeared outwardly healthy on examination and were given at least 8 days to acclimate prior to use in the study. Animals were identified by applying acrylic beads to the carapace using cyanoacrylate glue. They were fed a variety of frozen-thawed fish and shrimp three times weekly and fasted for 48 hr prior to anesthesia. Water quality parameters measured at least once weekly included pH (8.0) via colorimetric testing (API Salt Water Master Test Kit, Mars Fishcare, North America, Inc, Chalfont, PA 18914, USA), salinity (35–37 ppt) via refractometry, temperature (22.8°C–28°C), and

dissolved oxygen (DO, 4.5–6.8 mg/L) via a digital meter (YSI model 55 or 63, YSI, Yellow Springs, OH 45387, USA). Ammonia (0–0.25 ppm), nitrite (0.0 ppm), and nitrate (0.0 ppm) were measured initially and once weekly thereafter via colorimetric testing (Mars Fishcare). The North Carolina State University Institutional Animal Care and Use Committee regulations do not include invertebrates, but comparable standards of care were observed.

### Pilot study

Each of nine individuals was exposed to one of the following anesthetic treatments: MS-222 (TRICAINES, Western Chemical, Inc, Ferndale, WA 98248, USA) immersion at 0.5 or 1.0 g/L; 2-PE (Sigma-Aldrich, St Louis, MO 63103, USA) immersion at 1.0 or 2.0 mL/L; propofol (Propofol 28, Abbott Laboratories, North Chicago, IL 60064, USA) IV at 10, 20, or 30 mg/kg; and alfaxalone (Alfaxan, Jurox Inc, Kansas City, MO 64111, USA) IV at 10 or 20 mg/kg. Only MS-222 at 1 g/L and 2-PE at 2 mL/L produced anesthesia in the pilot animals. These animals were given a washout period of at least 14 days before the primary study.

### Procedures

Ten HSCs were randomly assigned (via lottery) to each of the two anesthetic treatment groups, MS-222 or 2-PE. They were anesthetized individually in tanks containing 30 L of seawater, an air stone, and a circulating pump. Water was changed between each anesthetic event. To minimize hemolymph gas changes associated with air exposure, the animals were placed in a tub containing seawater and an air stone for transport to the induction tank. To limit the influence of handling, all animals were given a 5-min acclimation period in the induction tank prior to obtaining baseline data. Water quality variables, including temperature, pH, salinity, and DO, were measured at  $T_0$  (prior to anesthesia) and at  $T_{30}$  (30 min into anesthesia). Physiologic variables measured at  $T_0$  and  $T_{30}$  included HR, GR, sedation score, fractional shortening (FS) via cross-sectional cardiac M-mode images, and hemolymph gas analysis. After obtaining  $T_0$  parameters, the anesthetic was added (30 g of MS-222 buffered with 14 g of sodium carbonate [Church & Dwight Co, Inc, Princeton, NJ 08540, USA] or 60 mL of 2-PE). HSCs were monitored every 5 min via HR, GR, and sedation score. After obtaining  $T_{30}$  measurements, they were transferred to recovery

tanks containing 30 L of anesthetic-free seawater, an air stone, and a circulating pump. They were monitored every 5 min via HR, GR, and sedation score until completely recovered (sedation score equal to baseline).

A sedation score was developed during the pilot study to characterize anesthetic depth via the evaluation of purposeful movement, response to tactile stimuli, righting reflex, and response to painful stimuli (Table 1).<sup>8</sup> The score was intended to discriminate between awake (score of 14), sedated (4–13), lightly anesthetized (2–3), and surgically anesthetized (0–1) individuals.

HR and GR were measured via ultrasonography (Sonosite M-series, FUJIFILM SonoSite, Inc, Bothell, WA 98022, USA). For HR measurements, the ultrasound probe was placed on the prosomal carapace immediately caudal to the compound eyes at midline to obtain a transverse image. For GR measurements, the ultrasound probe was placed on the left or right aspect of the opisthosomal carapace to obtain a longitudinal image of the book gills.

FS was determined from transverse M-mode echocardiogram images. Cardiac chamber width at systole and diastole were measured using the K-pacs Workstation (IMAGE Information Systems Ltd, Charlotte, NC 28210, USA). FS was calculated routinely (ventricular end diastolic dimension – ventricular end systolic dimension/ventricular end diastolic dimension) for three measurements, averaged, and reported as a percentage.

Hemolymph was collected from the cardiac sinus via the arthrodiol membrane at the prosomal-opisthosomal hinge using a 22-ga needle and 3-mL syringe. During venipuncture, the animals were restrained in slight ventroflexion and held partially submerged to maintain water contact with the book gills and limbs. Hemolymph was immediately placed in a CG8+ iStat cartridge (Abaxis, Union City, CA 94587, USA) and run using a portable point-of-care analyzer (VetScan iStat, Abaxis). Temperature-corrected values were based on the water temperature at the time of sample collection. Hemolymph gas values reported as <5 mm Hg were assigned a value of 5 to permit statistical analysis. Unmeasurable values were excluded from analysis. Percent hemocyanin saturation with oxygen (SO<sub>2</sub>) was approximated using oxygen-hemocyanin dissociation calculations published for HSC.<sup>22</sup>

Eight days after completion of the study, eight individuals were randomly chosen for collection of tank-side hemolymph gases to evaluate the

**Table 1.** Summary of criteria and scoring for assessment of anesthetic depth in American horseshoe crabs (*Limulus polyphemus*) (modified from Gjeltema et al<sup>8</sup>). The sedation score is the sum of the criteria scores.

Criteria	Description	Defined scores				
		0	1	2	3	4
Purposeful movement	Ambulation, limb movements, flexing body or telson	No movement	Minimal limb movements, no ambulation	Slow, uncoordinated movement	Slow, coordinated movements	Strong and coordinated
Response to tactile stimulation	Gentle traction applied to a single limb in ventral recumbency	No response, absent muscle tone	Muscle tone present, no withdrawal	Muscle tone weak or incomplete withdrawal	Strong muscle tone and withdrawal, no evasion	Evasive and coordinated
Righting response	Effort to right after placement in dorsal recumbency	No response, no limb movement	Weak limb movements and/or flexing	Placing telson on floor, unable to weight bear	Coordinated, telson weight bearing, lacks vigor	Vigorous attempts
Response to painful stimuli	Hemostat applied to arthrodiol membrane of fifth limb in dorsal recumbency	No response	—	Response	—	—

potential effect of transport and handling on the  $T_0$  partial pressure of carbon dioxide ( $p\text{CO}_2$ ) results. Animals were brought to the surface of their respective holding tanks and held partially submerged to maintain water contact with the book gills and limbs, and hemolymph was collected and immediately analyzed as above. Water quality parameters were obtained concurrently.

Two females not included in the primary study were used to obtain direct blood pressure measurements and to describe the effect of MS-222 and 2-PE on blood pressure. Each was assigned to one of the anesthetics and anesthetized in a tank containing 40 L of seawater, an air stone, and a circulating pump with the doses above. Invasive blood pressure monitoring, HR, GR, and FS measurements were performed at  $T_0$  and  $T_{30}$ . The animals were restrained partially submerged as for hemolymph collection. An 18-ga needle was introduced into the cardiac sinus and attached to a saline-filled low-compliance tubing connected to a commercial transducer (LogiCal Pressure Transducer, Smiths Medical OEM, Dublin, OH 43017, USA) positioned at the level of the heart and zeroed to atmospheric pressure. Intracardiac systolic, diastolic, and mean pressures were measured via an electronic monitor (Datascopie Spectrum Patient Monitor, Mindray, Mahwah, NJ 07430, USA). Blood pressure was evaluated for approximately 1 min at each time point. The animals were transferred to a tank containing anesthetic-free water for recovery.

An 8-day washout period was observed prior to release. All 22 animals were released at their sites of capture.

### Statistical analysis

Statistical analyses were performed using JMP Pro version 13.0 software (SAS Institute Inc, Cary, NC 27513, USA). Measured variables were tested for normality using the Shapiro-Wilk test. Because many data were nonnormally distributed, for consistency all data were either analyzed by nonparametric methods or transformed to ranks, as specified below. Paired data obtained at  $T_0$  and  $T_{30}$  (hemolymph values, water quality parameters, and FS) and weights at capture and release were compared using the Wilcoxon signed ranks test for matched pairs. Two-way comparisons between the treatment groups (morphologic data, hemolymph values, water quality, FS at  $T_0$  and  $T_{30}$ , time to induction, and time to recovery) and between sexes (time to induction, time to recovery) were performed using the Wilcoxon rank sums test. Repeated measures were evaluated for effects of

time and treatment group by transforming measurements to ranks (HR, GR, sedation score) and performing repeated-measures multivariate analysis of variance. Post hoc tests included Wilcoxon rank sums test for two-way comparisons and Wilcoxon signed ranks for paired data. A  $P$ -value of 0.05 was used to determine statistical significance. Corrected critical  $P$ -values for multiple comparisons were calculated using a Bonferroni adjustment and included water quality ( $P = 0.0125$ ), hemolymph gas analyses ( $P = 0.0125$ ), HR and GR ( $P = 0.005$ ), and sedation score ( $P = 0.0036$ ).

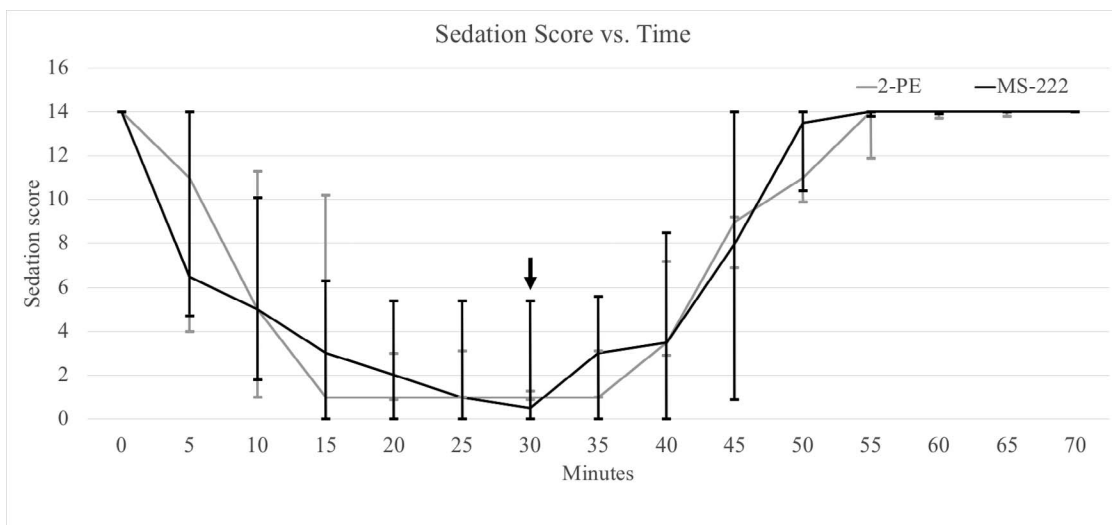
### RESULTS

HSCs weighed a median of 1.45 kg (0.82–3.18), and weights did not change significantly during the study. Weight, length, and sex did not differ significantly between the treatment groups. As is typical for this species, females were significantly heavier than males.

The median  $T_0$  water temperature was 27.6°C (25.4–29.4). Ambient air temperature was approximately 21°C. Water temperature was significantly decreased at  $T_{30}$  (median 26.7°C [25.6–28.8]) for both groups but did not differ between the groups at either time point. The median  $T_0$  salinity was 36 ppt (35–37), median  $T_0$  pH was 8.0 (8.0–8.2), and median  $T_0$  DO was 5.78 mg/L (4.72–7.59). Salinity, pH, and DO did not differ between  $T_0$  and  $T_{30}$  or between groups.

Sedation scores are presented in Figure 1. Light or surgical anesthesia was achieved by 10/10 animals in the 2-PE group and 8/10 animals in the MS-222 group. The remaining two animals reached minimum planes of moderate or deep sedation. Surgical anesthesia was achieved in 9/10 animals anesthetized with 2-PE and 7/10 animals anesthetized with MS-222. There was a significant effect of time but not treatment group on sedation score. Sedation score was significantly decreased from  $T_0$  at 5 through 50 min. Median time to induction (score of 3 or less) for the 18 animals that reached anesthesia was 15 min (5–20). Median recovery time was 20.5 min (12–36). There was no significant difference in time to induction or recovery between groups. Across both treatment groups, males exhibited a significantly faster recovery time compared to females (male median recovery 17 min [12–22]; female median recovery 25 min [17–36]).

HR is presented in Figure 2. There was a significant effect of treatment group but not time on HR. HR was significantly increased in the MS-222 group only at the 5-min time point. At 10 min,



**Figure 1.** Median sedation scores over time of American horseshoe crabs (*Limulus polyphemus*) anesthetized with MS-222 at 1 g/L (n = 10) or 2-PE at 2 mL/L (n = 10). Error bars represent the 10th and 90th percentiles. The arrow indicates the time placed in the recovery tank. Sedation scores did not differ significantly between the groups at any time point.

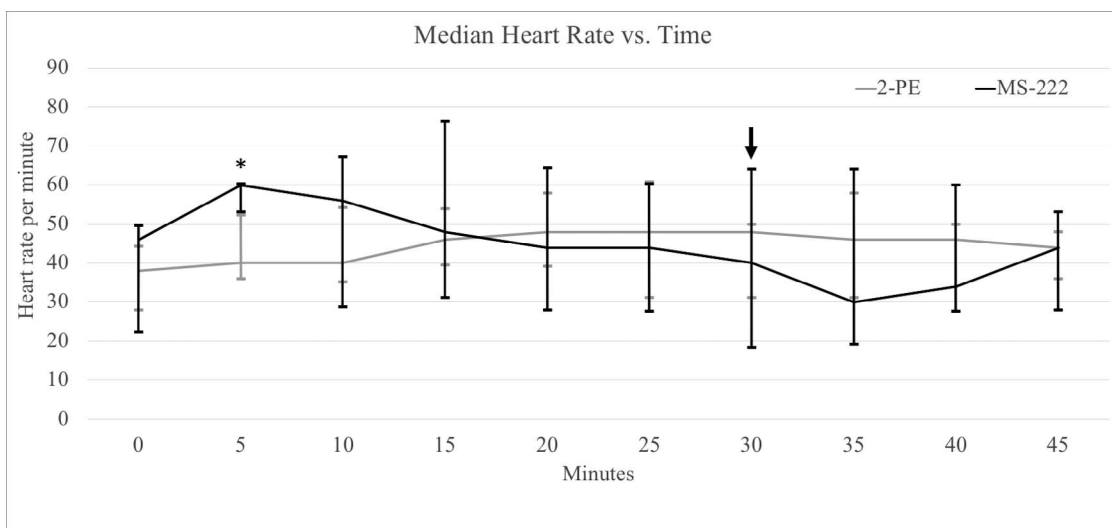
one animal exhibited rapid, uncoordinated myocardial contractions that could not be accurately counted.

GR is presented in Figure 3. There was a significant effect of time but not treatment group on GR. GR was significantly decreased from T<sub>0</sub> at 20, 25, and 30 min. As gilling decreased, the rate

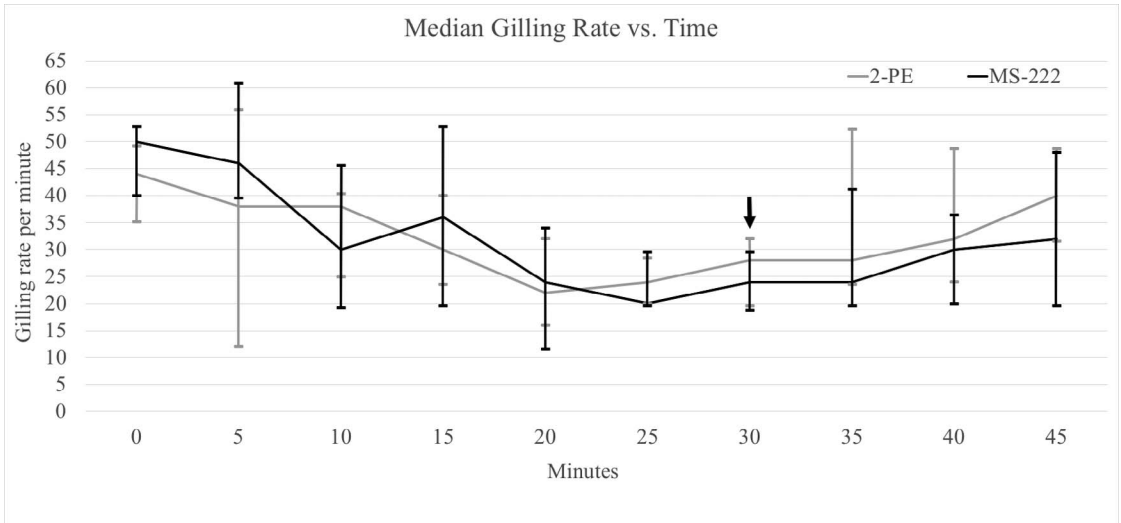
often became irregular, and the gill leaflet excursions appeared uncoordinated.

Percent FS is presented in Figure 4. FS was significantly decreased at T<sub>30</sub> in both groups but not different between the two treatment groups at either time point.

Blood pressure was measured via cardiac catheterization in two HSCs. The average intra-



**Figure 2.** Median heart rate (HR) over time of American horseshoe crabs (*Limulus polyphemus*) anesthetized with MS-222 at 1 g/L (n = 10) or 2-PE at 2 mL/L (n = 10). Error bars represent the 10th and 90th percentiles. The arrow indicates the time placed in the recovery tank. \*Heart rate was significantly elevated at 5 min in the MS-222 group compared to the 2-PE group.



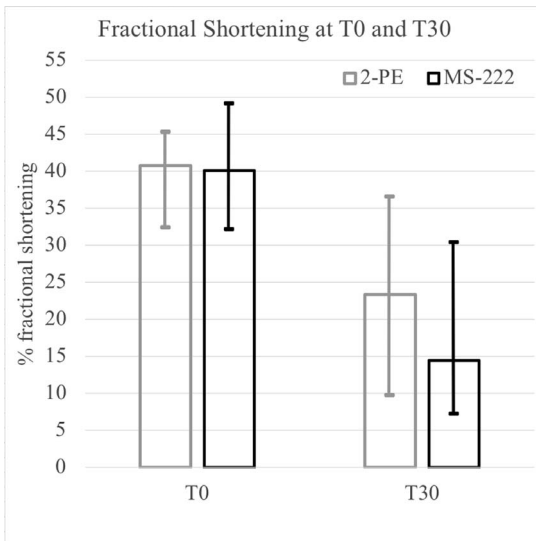
**Figure 3.** Median gilling rate (GR) over time of American horseshoe crabs (*Limulus polyphemus*) anesthetized with MS-222 at 1 g/L (n = 10) or 2-PE at 2 mL/L (n = 10). Error bars represent the 10th and 90th percentiles. The arrow indicates the time placed in the recovery tank. GR was significantly decreased from baseline at 20 to 30 min but did not differ between the groups at any time point.

cardiac pressures reported as systolic/diastolic (mean) at  $T_0$  for the MS-222 and 2-PE animals were 27/15 (20) and 27/5 (17) mm Hg, respectively. At 30 min into anesthesia, intracardiac pressures for the MS-222 and 2-PE animals were

1/0 (0) and 3/1(2) mm Hg, respectively. Both individuals reached surgical anesthesia and recovered uneventfully.

All pH,  $pCO_2$ , and bicarbonate ( $HCO_3$ ) values fell within the reportable ranges of the analyzer. Partial pressure of oxygen ( $pO_2$ ) at 37°C was reported as <5 mm Hg in six animals at  $T_0$  and two animals at  $T_{30}$ . Therefore, eight animals were assigned a  $pO_2$  5 mm Hg at 37°C to permit statistical analysis.  $pO_2$  was unmeasurable for one individual in the 2-PE group at  $T_0$ , and this individual was excluded from analysis. After temperature correction,  $pO_2$  was reported as <5 mm Hg or unmeasurable in nine animals at  $T_0$  and six at  $T_{30}$ . Therefore, the number of values for temperature-corrected  $pO_2$  was considered inadequate for statistical analyses. Hemolymph pH,  $pO_2$ ,  $pCO_2$ , and calculated  $SO_2$  at  $T_0$  and  $T_{30}$ , including tank-side values, are presented in Table 2. The table includes temperature-corrected values and those reported for 37°C.

Hemolymph  $HCO_3$ , pH,  $pCO_2$ , and  $pO_2$  at 37°C and temperature-corrected values did not differ significantly between groups at  $T_0$ . Median  $T_0$   $HCO_3$  was 2.9 mmol/L (range 1.4–4.3) and did not differ significantly at  $T_{30}$  or between groups. Calculated  $SO_2$  did not differ significantly between groups or time points. The pH and  $pO_2$  did not differ significantly between groups or time points for 37°C or temperature-corrected values. Median  $T_0$   $pCO_2$  values were higher than  $T_{30}$  for both groups, but this difference was statistically



**Figure 4.** Median percent fractional shortening (FS) of awake ( $T_0$ ) and anesthetized ( $T_{30}$ ) American horseshoe crabs (*Limulus polyphemus*) anesthetized with MS-222 at 1 g/L (n = 10) or 2-PE at 2 mL/L (n = 10). Error bars represent the 10th and 90th percentiles. FS was significantly decreased at  $T_{30}$  in both groups but did not differ between the groups at either time point.

**Table 2.** Hemolymph gas values at 37°C and temperature-corrected obtained from awake (T<sub>0</sub>) and anesthetized (T<sub>30</sub>) American horseshoe crabs (*Limulus polyphemus*) anesthetized with MS-222 at 1 g/L (n = 10) or 2-PE at 2 mL/L (n = 10) and awake tank-side without short transport (n = 8). Values include pH, partial pressure of carbon dioxide (pCO<sub>2</sub>), partial pressure of oxygen (pO<sub>2</sub>), and calculated percent hemocyanin saturation with oxygen (SO<sub>2</sub>) and represent a median and range of 10 animals unless otherwise specified.

Group	T <sub>0</sub>					T <sub>30</sub>						
	pH	pCO <sub>2</sub> mm Hg	pO <sub>2</sub> mm Hg	SO <sub>2</sub> %	pH	pCO <sub>2</sub> mm Hg	pO <sub>2</sub> mm Hg	SO <sub>2</sub> %	pH	pCO <sub>2</sub> mm Hg	pO <sub>2</sub> mm Hg	SO <sub>2</sub> %
<b>37°C</b>												
2-PE	7.01 (6.66–7.25)	10.9 (9.5–14.8)	19 (5–80) n = 9	96.6 (52.4–99.9) n = 9	7.03 (6.85–7.2)	10.45 (9.1–11.4)	10 (5–79)	78.5 (42–99.9)				
MS-222	7.04 (6.62–7.13)	10.95 (9.5–14.4)	17.5 (5–70)	91.5 (49.2–99.8)	7.01 (6.68–7.24)	9 (8.4–12.5)	33.5 (9–122)	99.2 (76.7–100)				
Tank-side	7.17 (7.14–7.23) n = 8	9.3 (8.4–10.9) n = 8	44 (8–103) n = 8	98.5 (56.4–99.9) n = 8	—	—	—	—	—	—	—	—
<b>Temperature corrected</b>												
2-PE	7.13 (6.76–7.31)	7.25 (6.1–9.7) <sup>a</sup>	17 (12–45) n = 5	—	7.15 (6.96–7.35)	6.65 (6.3–7.2)	5.5 (5–39) n = 8	—				
MS-222	7.15 (6.72–7.24)	7.2 (6.6–9.2) <sup>a</sup>	11.5 (5–33) n = 8	—	7.14 (6.79–7.4)	5.7 (5.1–8.5) <sup>b</sup>	16 (5–78)	—				
Tank-side	7.3 (7.27–7.36) n = 8	6.15 (5.5–7.1) <sup>b</sup> n = 8	23 (5–58) n = 8	—	—	—	—	—	—	—	—	—

<sup>a,b</sup> Medians in the same row and column with different superscripts are significantly different (P < 0.05).



significant only for temperature-corrected values in the MS-222 group. Median tank-side temperature-corrected  $p\text{CO}_2$  values were lower than  $T_0$  values. Tank-side  $p\text{CO}_2$  did not differ significantly from  $T_{30}$  values. The hemolymph pH was significantly higher in the tank-side samples compared to  $T_0$  for both 37°C and temperature-corrected values.

## DISCUSSION

Immersion in MS-222 at 1 g/L and 2-PE at 2 mL/L provided effective anesthesia in American HSCs and resulted in clinically useful induction and recovery times. Light or surgical anesthesia was produced in 100% of animals exposed to 2-PE and 80% exposed to MS-222. Despite a marked decrease in cardiac contractility noted in both groups, all animals exhibited smooth recoveries and no postanesthetic complications.

A physiologically significant drop in pH occurs when MS-222 is added to seawater at clinically useful concentrations, and thus buffering with sodium bicarbonate is routine in aquatic animal anesthesia.<sup>6</sup> In the present study, roughly 8 g of sodium bicarbonate per gram of MS-222 (240 g total) were needed to maintain a pH of 8.0, resulting in salinity >45 ppt. Sodium carbonate, a stronger buffer than sodium bicarbonate, was used in this study to maintain an appropriate pH with minimal effects on salinity. In the authors' experience, the amount of sodium carbonate added per gram of MS-222 to maintain a specific pH in a given volume is not a linear relationship and likely depends on the natural buffering capacity of the salt water used. When using MS-222 at the doses suggested here, the authors recommend titrating pH with a stepwise addition of sodium carbonate before introducing anesthetic patients.

There was no significant difference in sedation scores over time between the groups. Ninety percent of animals in the 2-PE group achieved surgical anesthesia compared to 70% in the MS-222 group. The animals that did not achieve surgical anesthesia had not been exposed to these anesthetics in the pilot study; consequently, tolerance is not a contributing factor, as has been reported in goldfish with repeated exposure to both MS-222 and 2-PE.<sup>21,34</sup> Interestingly, the individual exhibiting the least degree of sedation had a HR of 8 at  $T_0$ , the lowest of any awake animal in the study. Low cardiac output may have affected perfusion to the book gills and subsequent anesthetic uptake and systemic distribution.

Repetitive flexion and extension of the body and telson often occurred during the first 5 to 10 min of induction with MS-222. Compared to 2-PE, HR was elevated in the MS-222 group at 5 and 10 min. These findings suggest an excitatory phase of induction. In contrast, HSC anesthetized with 2-PE exhibited a subjectively smoother induction. In the 2-PE group, animals at surgical anesthesia often maintained minute, repetitive movements of the chelicera despite not responding to painful stimuli. The reason for faster recovery of male HSCs is unknown but may reflect a sexually dimorphic metabolism or an effect of body size, as males were significantly smaller than females.

There was a significant decrease in cardiac contractility but not HR over the course of anesthesia for both groups. This was observed as a dramatic decrease in the apparent strength of cardiac contractions visible ultrasonographically, with no appreciable change in frequency. These findings support the conclusion that contractility but not HR is indicative of anesthetic depth using these anesthesia protocols in HSCs. Research evaluating the origin of the HSC's cardiac impulse identified pacemaker-like cells within the median nerve cord (cardiac ganglion) running along the dorsal aspect of the heart.<sup>25</sup> There is no inherent spontaneous myocardial activity in HSCs, and if the median nerve cord is removed, contractile activity of the heart ceases. It is possible that the anesthetics in the current study differentially impacted the myocardium and median nerve cord, resulting in decreased contractility with no effect on HR. Decreased GR was associated with anesthetic depth and can therefore be useful for evaluating stage of anesthesia in this species. Additionally, sedated or anesthetized animals often exhibited irregular respiratory rates with uncoordinated gill leaflet excursions.

There was a dramatic decrease in blood pressure for the two animals evaluated. Blood pressure is a direct result of cardiac output and vascular resistance, both of which were potentially influenced by the anesthetics. A decrease in stroke volume, a primary determinant of cardiac output and correlate to FS, without a compensatory tachycardia, likely accounts for the marked hypotension reported here. HSCs exhibit low blood pressure in association with hypoxemia, and this also may have contributed to hypotension in the present study.<sup>23</sup> Additionally, HSC book gills have been described as a "second heart," acting to pump blood back to the pericardial sinus during each leaflet excursion.<sup>33</sup> It is possible that decreased and irregular gilling compromised

vascular return, thereby exacerbating low intra-vascular pressures. Despite confirmed hypocontractility and apparent hypotension occurring with both anesthetics, all the HSCs recovered within a clinically useful time frame and did not show any overtly negative effects.

Hemolymph gas values obtained with the iStat point-of-care analyzer have been evaluated in HSCs,<sup>1</sup> but this analyzer has not been validated in this species. The analyzer calculates several variables using algorithms developed for mammals, likely precluding meaningful measurements in HSCs. Namely, base excess is calculated at 37°C using the ideal pH and pCO<sub>2</sub> values of 7.4 and 40 mm Hg, respectively; however, published baseline hemolymph pH and pCO<sub>2</sub> values in HSCs are lower than mammals.<sup>1</sup> HSC hemolymph does not contain red blood cells or hemoglobin. Therefore, hematocrit, hemoglobin, and base excess were not reported, and temperature corrections should be regarded qualitatively and comparatively rather than as definitive representations of HSC physiologic status. Additionally, the analyzer calculates SO<sub>2</sub> using pO<sub>2</sub>, pH, and HCO<sub>3</sub> by assuming normal affinity of oxygen to hemoglobin. Hemocyanin exhibits markedly dissimilar oxygen-binding characteristics compared to hemoglobin; therefore, previously published HSC-specific oxygen-hemocyanin dissociation characteristics were used to calculate SO<sub>2</sub>.<sup>22</sup> However, the laboratory conditions in which the curves were established vary slightly from those of the current study, and thus the calculated values are only approximations.

The hemolymph pH of the study animals did not differ between treatment groups or time points but was lower than previously published for captive HSCs.<sup>1</sup> Possible causes include a respiratory or metabolic acidosis associated with handling of wild-caught animals or the influence of water quality on hemolymph parameters. However, pCO<sub>2</sub> was similar to the previously reported baseline values, and water quality variables were comparable between studies.<sup>1</sup> It is unknown if acidic metabolites such as lactate contributed to a relative acidemia. Two previous investigations were unable to detect L-lactate in HSCs using mammalian benchtop or point-of-care analyzers.<sup>1,10</sup> In contrast to the predominant isoform found in mammals (L-lactate), HSCs are reported to have D-specific lactate dehydrogenases and are therefore presumed to produce D-lactate.<sup>31</sup> Total lactate concentration, measured in HSCs using laboratory techniques, increased in association with decreased DO exposure and

decreased hemolymph pH.<sup>31</sup> No commercially available benchtop or point-of-care analyzers detect the D isoform. Interestingly, HSCs and other arthropods are believed to produce succinate as a primary product of anaerobic metabolism.<sup>7</sup> Succinate may therefore represent an unmeasured contributor to metabolic acidemia.

Hemolymph pCO<sub>2</sub> values were similar to previous reports.<sup>1</sup> Unexpectedly, HSCs in the current study had higher pCO<sub>2</sub> values awake (T<sub>0</sub>) than under anesthesia (T<sub>30</sub>) despite decreased ventilation during anesthesia. The T<sub>0</sub> hemolymph samples were collected following transport to the induction tank and after the completion of baseline sedation scoring. Both events involved direct manipulation of the animals and appeared to cause increased activity. Tank-side pCO<sub>2</sub> values were lower than T<sub>0</sub> and did not differ significantly from T<sub>30</sub>, suggesting that the response to handling and transport may have resulted in hypercapnia at T<sub>0</sub>.

Hemolymph pO<sub>2</sub> was markedly variable at T<sub>0</sub> and T<sub>30</sub> for both groups and in tank-side samples. A previous study also reported a wide variation in pO<sub>2</sub>, though with a significant decrease in pO<sub>2</sub> following 5 min of air exposure, suggesting that the iStat can detect clinically meaningful changes in blood-oxygen tensions in this species.<sup>1</sup> The cause of variable pO<sub>2</sub> is unknown. Possibilities include transient hypoxia associated with the handling, proximity to the circulating pump, behavioral apnea and/or bradycardia at the time of hemolymph collection, influence of pH on oxygen-binding properties of hemocyanin, or unidentified interference with the analyzer.

## CONCLUSIONS

Immersion in 2-PE or MS-222 produced anesthesia in American HSCs with clinically useful induction and recovery times. 2-PE provided a subjectively more reliable, smoother induction and, in contrast to MS-222, does not require the addition of buffers and is less expensive per dose. Transcarapacial ultrasonography was an excellent anesthesia monitoring tool. GR and cardiac contractility but not HR can be used as indicators of anesthetic depth. Marked changes in cardiac contractility and blood pressure may preclude the use of these protocols for prolonged anesthetic events. These effects could be mitigated by using lower doses for anesthesia maintenance following induction. Anesthesia did not appear to have a clinically significant effect on hemolymph pH or pCO<sub>2</sub>. Direct effect of the anesthetics on hemolymph pO<sub>2</sub> was difficult to determine due to wide

variability. The anesthesia protocols in this study can be used for invasive diagnostic procedures or as a component of euthanasia. These findings expand the literature relating to HSC anesthesia and will help improve the veterinary management of this unique species.

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### LITERATURE CITED

- Allender MC, Schumacher J, George R, Milam J, Odoi A. The effects of short-and long-term hypoxia on hemolymph gas values in the American horseshoe crab (*Limulus polyphemus*) using a point-of-care analyzer. *J Zoo Wildl Med.* 2010;41(2):193–200.
- Allender MC, Schumacher J, Milam J, George R, Cox S, Martín-Jiménez T. Pharmacokinetics of intravascular itraconazole in the American horseshoe crab (*Limulus polyphemus*). *J Vet Pharmacol Ther.* 2008; 31(1):83–86.
- Braverman H, Leibovitz L, Lewbart GA. Green algal infection of American horseshoe crab (*Limulus polyphemus*) exoskeletal structures. *J Invertebr Pathol.* 2012;111(1):90–93.
- Brouwer M, Bonaventura C, Bonaventura J. Chloride and pH dependence of cooperative interactions in *Limulus polyphemus* hemocyanin. In: Bonaventura J, Bonaventura C, Tesh S (eds.). *Progress in clinical and biological research, Volume 81, Physiology and biology of horseshoe crabs: studies on normal and environmentally stressed animals.* New York (NY): A. R. Liss; 1982. p. 231–256.
- Chesmore KN, Watson WH, Chabot CC. Identification of putative circadian clock genes in the American horseshoe crab, *Limulus polyphemus*. *Comp Biochem Phys D.* 2016;19:45–61.
- Christiansen EF, Stoskopf MK. pH dynamics of tricaine methanesulfonate (MS-222) in fresh and artificial seawater. *N Am J Aquacult.* 2013;75(3):356–360.
- Fields JH. Anaerobiosis in *Limulus*. In: Bonaventura J, Bonaventura C, Tesh S (eds.). *Progress in clinical and biological research, Volume 81, Physiology and biology of horseshoe crabs: studies on normal and environmentally stressed animals.* New York (NY): A. R. Liss; 1982. p. 125–131.
- Gjeltema J, Posner LP, Stoskopf MK. The use of injectable alphaxalone as a single agent and in combination with ketamine, xylazine, and morphine in the Chilean rose tarantula, *Grammostola rosea*. *J Zoo Wildl Med.* 2014;45(4):792–801.
- Iwanaga S. The molecular basis of innate immunity in the horseshoe crab. *Curr Opin Immunol.* 2002; 14(1):87–95.
- James-Pirri M, Veillette PA, Leschen AS. Selected hemolymph constituents of captive, biomedically bled, and wild caught adult female American horseshoe crabs (*Limulus polyphemus*). *Mar Freshw Behav Physiol.* 2012;45(4):281–289.
- Kirby A, Lewbart GA, Hancock-Ronemus A, Papich MG. Pharmacokinetics of enrofloxacin and ciprofloxacin in Atlantic horseshoe crabs (*Limulus polyphemus*) after single injection. *J Vet Pharmacol Ther.* 2018;41(2):349–353.
- Kwan BK, Chan AK, Cheung SG, Shin PK. Hemolymph quality as indicator of health status in juvenile Chinese horseshoe crab *Tachypleus tridentatus* (Xiphosura) under laboratory culture. *J Exp Mar Biol Ecol.* 2014;457:135–142.
- Leibovitz L, Lewbart GA. Diseases and symbionts: vulnerability despite tough shells. In: Shuster CN, Brockmann JH, Barlow RB (eds.). *The American horseshoe crab.* Cambridge (MA): Harvard University Press; 2003. p. 358–377.
- Liu JS, Passaglia CL. Using the horseshoe crab, *Limulus polyphemus*, in vision research. *Jove-J Vis Exp [Internet].* 2009 [cited 2018 February 18];29:1–5. Available from doi:10.3791/1384
- Mitjana O, Bonastre C, Insua D, Falceto MV, Esteban J, Josa A, Espinosa E. The efficacy and effect of repeated exposure to 2-phenoxyethanol, clove oil and tricaine methanesulfonate as anesthetic agents on juvenile angelfish (*Pterophyllum scalare*). *Aquaculture.* 2014;433:491–495.
- Mori K, Saito T, Barlow RB. Processing of retinal signals in the *Limulus* brain. In: *Proc International Conference on Intelligent Informatics and Biomedical Sciences;* 2015. p. 300–307.
- Mosley CL, Mosley CA. Comparative anesthesia and analgesia of reptiles, amphibians, and fishes. In: Grimm KA, Lamont LA, Tranquilli WJ, Greene SA, Robertson SA (eds.). *Veterinary anesthesia and analgesia.* 2nd ed. Ames (IA): Wiley Blackwell; 2015. p. 784–799.
- Nolan MW, Smith SA. Clinical evaluation, common diseases, and veterinary care of the horseshoe crab, *Limulus polyphemus*. In: Tanacredi JT, Botton ML, Smith DR (eds.). *Biology and conservation of horseshoe crabs.* New York (NY): Springer; 2009. p. 479–499.
- Nolan MW, Smith SA, Jones D. Pharmacokinetics of oxytetracycline in the American horseshoe crab, *Limulus polyphemus*. *J Vet Pharmacol Ther.* 2007;30(5): 451–455.
- Pathak A, Singh SK, Thirumalai A, Armstrong PB, Agrawal A. Evolution of host-defense function of C-reactive protein from horseshoe crab to humans. *J Immunol.* 2016;196(1):132–135.
- Posner LP, Scott GN, Law JM. Repeated exposure of goldfish (*Carassius auratus*) to tricaine methanesulfonate (MS-222). *J Zoo Wildl Med.* 2013;44(2):340–347.

22. Redfield AC, Ingalls EN. The oxygen dissociation curves of some bloods containing hemocyanin. *J Cell Comp Physiol.* 1933;3(2):169–202.
23. Redmond JR, Jorgensen DD, Bourne GB. Circulatory physiology of *Limulus*. In: Bonaventura J, Bonaventura C, Tesh S (eds.). *Progress in clinical and biological research, Volume 81, Physiology and biology of horseshoe crabs: studies on normal and environmentally stressed animals.* New York (NY): A. R. Liss; 1982. p. 133–146.
24. Ross LG, Ross B. Anaesthesia of aquatic invertebrates. In: Ross LG, Ross B (eds.). *Anaesthetic and sedative techniques for aquatic animals.* 3rd ed. Ames (IA): Blackwell; 2008. p. 167–178.
25. Shuster CN. The circulatory system and blood of the horseshoe crab. Washington (DC): US Department of Energy, Federal Energy Regulatory Commission, Office of Regulatory Analysis (DOE/FERC/0014); 1978. 63 p.
26. Shuster CN, Botton ML, Loveland RE. Horseshoe crab conservation: a coast-wide management plan. In: Shuster CN, Brockmann JH, Barlow RB (eds.). *The American horseshoe crab.* Cambridge (MA): Harvard University Press; 2003. p. 358–377.
27. Smith DR, Beekey MA, Brockmann HJ, King TL, Millard MJ, Zaldívar-Rae JA. *Limulus polyphemus*. The International Union for Conservation of Nature (IUCN) Red List of Threatened Species [Internet]. 2016 [cited 2018 February 2]. Available from <http://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T11987A80159830.en>
28. Smith DR, Brockmann HJ, Beekey MA, King TL, Millard MJ, Zaldívar-Rae J. Conservation status of the American horseshoe crab (*Limulus polyphemus*): a regional assessment. *Rev Fish Biol Fish.* 2017;27(1): 135–175.
29. Smith SA. Horseshoe crabs. In: Lewbart GA (ed.). *Invertebrate medicine.* 2nd ed. Ames (IA): Wiley-Blackwell; 2011. p. 173–185.
30. Towle DW, Henry RP. Coping with environmental changes: physiological challenges. In: Shuster CN, Brockmann JH, Barlow RB (eds.). *The American horseshoe crab.* Cambridge (MA): Harvard University Press; 2003. p. 224–244.
31. Towle DW, Mangum CP, Johnson BA, Mauro NA. The role of the coxal gland in ionic, osmotic, and pH regulation in the horseshoe crab *Limulus polyphemus*. In: Bonaventura J, Bonaventura C, Tesh S (eds.). *Progress in clinical and biological research, Volume 81, Physiology and biology of horseshoe crabs: studies on normal and environmentally stressed animals.* New York (NY): A. R. Liss; 1982. p. 147–172.
32. Tuxbury KA, Shaw GC, Montali RJ, Clayton LA, Kwiatkowski NP, Dykstra MJ, Mankowski JL. *Fusarium solani* species complex associated with carapace lesions and branchitis in captive American horseshoe crabs *Limulus polyphemus*. *Dis Aquat Organ.* 2014;109(3):223–230.
33. Watson WH, Groome JR. Modulation of the *Limulus* heart. *Am Zool.* 1989;29(4):1287–1303.
34. Weyl O, Kaiser H, Hecht T. On the efficacy and mode of action of 2-phenoxyethanol as an anaesthetic for goldfish, *Carassius auratus*, at different temperatures and concentrations. *Aquac Res.* 1996;27(10):757–764.

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