Housing, Husbandry, and Clinical Techniques for Crayfish (*Procambarus clarkii*) used in Biomedical Research

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Abstract

Crayfish (Decapoda: Astacoidea & Parastacoidea), are amongst the few animals that have stem cells within hemolymph, with the capacity to continuously produce differentiated neuronal structures throughout life. As crayfish and other invertebrates continue to become common models in research to study human disease, it is vital that we develop universal laboratory standards and guidelines on housing and husbandry practices. This publication presents introductory data on housing, husbandry, hemolymph collection and statistically supported anesthesia trials, to support future research endeavors. Evaluation of housing, husbandry, clinical and anesthetic techniques in *Procambarus clarkii* maintained in a biomedical research setting were performed. An option for hemolymph collection, in the area termed, the *Ventral Coelomic Hemolymph Collection Zone (VCHCZ)* is presented as a technique to assess hemocytes. Additionally, Wright Giemsa stained slides of crayfish hemolymph were evaluated by a board-certified veterinary clinical pathologist for interpretation and confirmation of hemolymph collection.

The housing and husbandry experiments were performed over a duration of 37 days. Mortality rates and physical health assessments were performed. The following water quality parameters were concurrently evaluated: temperature, light cycle, pH, KH, GH, conductivity, total dissolved solids, salinity, ammonia, nitrite, and nitrate.

Anesthetic techniques were evaluated between four experimental groups: (A) immersion in buffered MS-222 (50 mg/L), (B) immersion in buffered MS-222 (150 mg/L) (C) immersion in Propofol (65 mg/L) and (D) Propofol injection (100 mg/kg) into VCHCZ. Housing and husbandry techniques were validated with 0% mortality and normal species-specific behaviors were observed. MS-222 immersion had no observable effect on crayfish. Propofol immersion (65 mg/L) created sedative effects allowing for appropriate handling. Propofol injection (100 mg/kg) into VCHCZ successfully created a deep anesthetic plane that would allow for more invasive or surgical procedures, without adverse effects during or after recovery.

Introduction

Crayfish are freshwater crustaceans that have become increasingly popular in epigenetic, microbiome, stem cell and evolutionary biology research. [14,19,102,103,110,114,37,40,42,82,87,94,100,101] This is primarily due to *Procambarus spp* small size, high fecundity, and overall environmental and nutritional adaptability. [40,78,82,101] Additionally, there is clear potential for crayfish to become a mainstay neuroregenerative and immunologic animal model. [84,92,100] As of late, researchers have found that *Procambarus clarkii (Red Swamp Crayfish)* has the unique ability to continuously produce neuronal structures, throughout adulthood, through selective differentiation of innate immune cells, acting as neural precursors. [14] Additionally, these systems provide regenerative abilities to sensory organs and central nervous structures, essentially providing an unparalleled model for “neuronal” regeneration. [19,22,92] Historically, the crayfish genus *Procambarus* has significantly contributed to the aquaculture industry globally and has proven to be a vital mainstay to the economy, responsible for $59 million dollars a year in revenue for the state of Louisiana, USA alone. [57,89] The widespread availability with which *P. clarkii* can be obtained from aquaculture producers makes this species easily obtained and promising as a model organism for biomedical research. As use increases, opportunities will arise to create biomedical *P. Clarkii* colonies within biomedical vivaria for inter-institutional use. Furthermore, as *Procambarus virginalis* (clonal crayfish) becomes increasingly more available as a biological model, it is important to categorize husbandry parameters for this genus to increase validity and decrease confounding factors for investigators. [40,43,62,104]

Nonhuman primate model use has decreased in recent years due to legislation and public opinion in both Europe and the United States. [21,53] In 2021, the most popular animal model was the rodent, estimated at 111.5 million utilized in research.[20] As science progressed, the transgenic animal model revolutionized rodent research, making them even more popular, but even so, rodents still have their limitations.[51,58] As we have hit roadblocks using rats, mice, and nonhuman primates, it is important to explore new, alternative animal models to continue to make scientific advancements.[15,21,53]

Aquatic animal models are becoming more popular due to the ability to control precise environmental variables, giving insight into xenobiotic chemicals and mechanisms.[37] *Danio rerio (*Zebrafish) have proven to be invaluable in advancing a myriad of research areas including carcinogenesis, tissue regeneration, genetic disease and disorder pathogenesis, gerontology, and hematopoiesis.[29,36,37,50,55,70,98,101,108]Like zebrafish, crayfish have the potential to become an important research model, and it is therefore vital for animal research facilities to develop protocols on how to efficiently maintain these animals in a humane manner.

In the event that a facility does not have the capabilities to house crayfish in commercial production or laboratory aquaculture systems, it is important for laboratory animal professionals to provide alternative indoor life support systems. Although they are currently not a covered species in common regulatory documents within the USA, our group investigated housing, husbandry, and clinical techniques that would provide important information to most federal research governing agencies and groups (i.e., USDA, AAALACi, USDA, OLAW).

In this study the authors investigated housing systems, husbandry protocols, animal health and clinical techniques for laboratory housed crayfish (*Procambarus clarkii*). We also presented an approach for coelomic hemolymph collection technique, at the *Ventral Coelomic Hemolymph Collection Zone (VCHCZ)*, as an additional collection site for crayfish. We hypothesized that our life support system and husbandry techniques will provide a safe environment for the laboratory housed *Procambarus* crayfish and promote normal species-specific behaviors. We additionally hypothesized that both propofol and tricaine methanesulfonate (MS-222) would be efficacious anesthetic agents in laboratory housed *P. clarkii*, while propofol injection directly into the VCHCZ would yield the fastest onset and duration of anesthesia, as well as, slowest recovery time.

Materials And Methods

Housing and Husbandry Experiment

Animals. The study used 24 sexually mature, adult, farm-raised crayfish *(P. clarkii)*. Crayfish were maintained in an AAALACi accredited, USDA-registered, OLAW-assured facility (The Ohio State University) on a 12:12-h light cycle. 12 female and 12 male *P. clarkii* (*n* = 24), with an average weight of 14.88 grams (range: 10–23 grams), were included in the study. All animals were obtained from a commercial crayfish producer (Louisiana Crawfish Co, Natchitoches, LA, USA) and shipped overnight to ensure minimal transit time. Debris and detritus material were removed through three freshwater baths with water as described in the *Water Preparation* section below.

Life Support System. Crayfish housing consisted of a primary containment system, substrate, stress reduction hide, basic biological filter and air source. Systems were considered static system with individual tank aeration and basic bio-filtration. The 24 crayfish used, all had identical setups to promote efficacy and reproducibility of this system, while decreasing confounding factors. All life support systems were set up four weeks prior to introduction of the crayfish, to allow for proper cycling of the systems. Beneficial bacteria (Microbe-Lift Special Blend Water Care, Ecological Laboratories Inc, Cape Coral, FL, USA) were added as per the manufacturer's instructions to help hasten the cycling of each individual tank.

Primary containment system. Clear polypropylene plastic containers (Our Shoe Box, Container Store, Coppell, TX, USA) were used to individually house *P. clarkii.* These containment systems (33cm x 19cm x 11.5cm) were large enough to hold a minimum of 3.25 L of water and to contain our *P. clarkii*, while giving them appropriate space to explore the enclosure. The containers include clip-locking lids. Modification of the lids included two holes that were drilled in different aspects of the lid to support data collection and

basic biological filtration setup. The first was a 6.35mm (0.25 inch) hole, drilled into the towards the back of the lid on midline, enabling 4.76mm (3/16th inch) airline tubing (Elite Silicon Airline Tubing, Marina Products, Hagan, Montreal,

Quebec, Canada) to be passed into the internal environment. The second was a 12.7mm (0.5 inch) hole drilled into the front left aspect of the lid, enabling a 6 ml syringe to rest on the lid while still in the internal environment, allowing for water collection without overt disruption of the crayfish environment.**(Fig. 1C, O white).**

Stress reduction hide. Two-inch diameter white PVC tubing cut into a length of 4 inches served as a reclusive area for crayfish to take shelter. This allowed for performance of natural species-specific behaviors such as a safe environment while eating and hiding while resting **(Fig. 1C, Δ white).**

Air source. Two tetra whisper air pumps rated for 227 liters (60 gallons) (Tetra, Blacksberg, VA, USA) were used to provide air to each of the 24 individual tanks (One air pump per 12 tanks or 24 liters). Each of the air pumps had two outlets for aquarium grade silicone airline tubing. In an effort to enable these pumps to adequately supply air to 12 tanks, two six-way air flow aquarium lever valves (Uxcell, Hong Kong, China) were attached inline from each of the two air pump outlets allowing for equal division of airflow to each respective tank **(Fig. 1B,).**

Basic Biological filter. Aquarium grade, cylindrical, pre-filter foam (HuYaYa Tech, Seattle, WA, USA) was arranged so that it sat within the distal aspect of the stress reduction hide with the silicon airline fixed in the center of the foam **(Fig. 1C, O white).** This allowed for water to flow from the proximal aspect of the containment system, through the stress reduction hide, and through the foam **(Fig. 1C,).** This was a replication of a basic foam filter, custom fit for this setup.

Substrate. Two cups of pea gravel pebbles (Vigoro 0.5 cu ft bagged, Home Depot, Atlanta, GA, USA) were placed at the bottom of each primary containment system. Prior to use, the pea gravel was thoroughly washed with facility water, to remove any debris **(Fig. 1C white).**

Water acquisition. Water used within the life support systems was obtained from the research facility. The facility vivarium utilizes a reverse-osmosis water purification system to ensure removal of chemicals and pathogenic microbes (Indigo RO™ Reverse Osmosis System, Avidity Science, Long Credon, Aylesbury HP18 9BD, UK). The system is maintained through vivarium and university management. The water was treated with chlorine after osmosis to help ensure adequate microbe removal within the facility. Due to this, a commercial aquarium dechlorinator (API, Mars Fishcare, Chalfont, PA, USA) was added to the water after collection before being utilized, as per manufacturer instructions. Verification of chlorine removal was not validated, but water was left to sit for 24 hours before being utilized for the experiment to help promote dissolution of chlorine.

Water Preparation. The nature of reverse-osmosis creates water that is void of the natural anions and cations needed to sustain life for crayfish and other aquatic species. To rectify this issue, 2.5 ml of Replenish™ (Seachem Laboratories, Madison, GA, USA) was added to every 5 gallons of facility water.

Husbandry and Water Quality Testing. 30 mL of water was removed each day for water quality testing. This water was replaced daily with premade, conditioned water, as explained in the *Water Acquisition* and *Water Preparation sections*. Every seven days, routine maintenance of the containment systems included removal of 50% of the water. This occurred through syphoning the tank with 4.76mm (3/16th inch) airline tubing (Elite Silicon Airline Tubing, Marina Products, Hagan, Montreal, Quebec, Canada), until 1.6 L of water was removed. This water then was replaced, with conditioned water, as explained in the *Water Acquisition* and *Water Preparation sections*, to dilute nitrate, nitrate and waste products. Water quality testing occurred daily from day 0 to day 15, and then again on day 37. *Apera PC60-Z Smart Multi-Parameter Pocket Tester* (Apera Instruments LLC, Columbus, OH, USA) was used to measure temperature (°F), pH, conductivity (mS/cm), total dissolved solids (ppm) and salinity (ppt). *API Freshwater Master Test Kit* (MARS Fishcare, Chalfont, PA, USA) was used to perform ammonia (ppm), nitrite (ppm) and nitrate (ppm) testing. *API GH & KH Test Kit* (MARS Fishcare, Chalfont, PA, USA) was used to measure KH (ppm) and GH (ppm). All tests were performed in concordance with the manufacturer's instructions and reagents kept up to date.

Feed and Feeding Frequency. Crayfish were fed once a week. They received 3 pellets (0.5-1 gram), of a high quality shrimp pellets (Wardley-Hartz, Hartz Mountain Industries, Secaucus, NJ, USA). Guaranteed Analysis included 36% Crude Protein, 8% Crude Fat, and 36.5% Crude Fiber. Carbohydrate analysis was not available upon request. Any remaining food or debris was left until weekly water changes were performed.

Physical Health Assessments. During the initial addition of *P. clarkii* to the housing system, the physical health of each crayfish was assessed by a member of the study team, through the use of the *Red Swamp Crayfish Health Assessment Scale* (RSCHA)**(Fig. 2)**. These assessments focused on the following structures of the crayfish: antennae, antennules, maxillipeds, carapace, abdomen, telson, uropod, eyes, rostrum, and pereopods. Each structure was given one of five possible scores based on its current condition: 1, structure missing; 0.75, major damage; 0.5, medium damage; 0.25, minor damage; 0, no damage. The assignment of a particular score to a damaged structure was subjective but based on significant past experience with crayfish husbandry. Most minor and medium damage consisted of shell rot (i.e., melanization of injuries) to varying degrees. Scores were summed to get an overall idea of physical health of each crayfish, with zero indicating no damage, and 13 indicating catastrophic physical damage. Further reading for anatomic identification is encouraged through Holdich 2002.[38]

Hemolymph Collection

Restraint and Hemolymph Collection at the Ventral Coelomic Hemolymph Collection Zone. Prior to hemolymph collection each crayfish was weighed. Sterile 25G Precision Glide™ Needle (BD, Franklin Lakes, NJ, USA) and 1mL Insulin Syringe with Slip Tip (BD, Franklin Lakes, NJ, USA) were utilized for this procedure. Current vertebrate nonterminal bleeding standards in mammals, limit blood draws to 10% of total circulating volume.[72,73] The crayfish were held in dorsal recumbency. Careful restraint was implemented to ensure the proximal aspect of both chelipeds(claws) were contained within the study team member's thumb and index finger to prevent injury to the crayfish or the study team **(Fig. 3A & 3B)**. Landmarks used to determine needle entry site for hemolymph collection included: the anterior margin of the third abdominal somite, cranially; the posterior margin of the sixth abdominal somite, caudally; and the lateral aspects of each somite. This area we have termed the *Ventral Coelomic Hemolymph Collection Zone (VCHCZ)*(Figs. 3C & 4B). The needle was inserted in the intersegmental membrane, bevel up, 1–2 cm lateral to midline to ensure that no damage was done to the ventral nerve cord. The needle should be inserted at a 30° angle relative to the frontal plane of the crayfish to a depth of approximately 1 cm to avoid damaging musculature or neurological structures. Gentle negative pressure should be applied to the syringe once inserted, to remove hemolymph from the crayfish. Collected hemolymph samples were placed in a Vacutainer EDTA anticoagulant tube (BD, Franklin Lakes, NJ, USA) and a Vacutainer Red top tube (BD, Franklin Lakes, NJ, USA). The samples were submitted to The Ohio State University, Clinical Pathology Lab, within the Department of Veterinary Biosciences for further interpretation through peripheral blood smear microscopic evaluation with Wright Giemsa staining for verification of hemolymph collection.

Hemolymph Preparation. Direct smears and cytocentrifuged samples of the hemolymph were made. Cellular morphology of the hemocytes were evaluated using the cytocentrifuged slides because of the viscosity of the hemolymph. Cytocentrifugation is a technique commonly used in veterinary medicine to evaluate samples that have a low cellularity; however it is also useful in the evaluation of highly viscous samples. In highly viscous samples, cells tend to constrict which makes evaluation of cytoplasmic and nuclear features impossible. Cytocentrifugation of samples results in the flattening of cells so that they adhere to the slide; the flattening of cells also improves visualization of cytoplasm and nuclear detail.[90]

Anesthesia Experiments

Anesthesia Assessment. To assess the time to anesthesia, *P. clarkii* tactile function was observed after administration of anesthetic agent. Qualitative anesthesia scores were created. Stage of anesthesia was adapted from published work by

Coyle et al 2004.[23] Assessment of depth of anesthesia was adapted from sedation score tables published by Archibald et al 2019 for *Limulus polyphemus* anesthesia.[10]

Surgical anesthetization was considered: little to no purposeful limb movement; minimal to absent muscle tone present with no withdrawal reflex in response to tactile stimulation; no righting response with absent to weak limb movement; no response to painful stimuli through hemostat applied to arthrodial membrane of the second limb while in dorsal recumbency. *Sedation* was considered: slow uncoordinated to coordinated limb movement; response to tactile stimulation with muscle tone present and weak to absent evasion/withdrawal; coordinated righting reflex with telson bearing weight; and response to painful stimuli through digital applied pressure to arthrodial membrane of the second limb in dorsal recumbency. An *awake crayfish* was considered: Normal purposeful movement; strong evasion of tactile stimulation; and vigorous righting reflex.

MS-222. MS-222 (Tricaine-S topical anesthetics, Syndel Laboratories, Ferndale, WA, USA) was tested in a time and dose-dependent immersion design; at 50 and 150 mg/L. Stock solutions were made the day of the study by dissolving MS-222 in conditioned facility water (see *Water Preparation* section). To account for the acidic nature of MS-222, medical grade sodium bicarbonate (Sodium Bicarbonate 8.4% Injection, VetOne, MWI, Boise, ID, USA) was added to the stock solution until the stock solution measured a pH of 7.0, validated through an *Apera PC60-Z Smart Multi-Parameter Pocket Tester.* For each of the doses, three 113.6L totes (Sterilite, 30-gallon [120 quarts] Lowes, Mooresville, NC, USA) were filled with 4L of stock solution. Two cohorts of crayfish were used, one for each dose of MS-222 tested. Each cohort included 16 crayfish (8 males, 8 females; weight range: 11.8-21.6g), independent of all other study arms. Crayfish were separated while in stock solution through the use of porous plastic baskets, (Y-Weave Cube Storage Basket, 2”X6”, Target, Minneapolis, MN, USA) placed in the larger 113.6L totes, to prevent crayfish interacting with each other. This setup similar to **Fig. 4A**. The 50 mg/L cohort had an 120 minute exposure time. The 150mg/L cohort had a 90 minute exposure time. Time to anesthesia and any changes in behavior were recorded. Following MS-222 exposure, crayfish were placed in pre-conditioned water for a 24 hour recovery period.

Propofol. The effect of Propofol (PropoFlo, Zoetis, Parsippany-Troy Hills, NJ, USA) on *P. clarkii* was assessed through a route of administration and time study design protocol.

Propofol immersion stock solution was prepared by diluting propofol in conditioned facility water (see *Water Preparation* section) until the solution had a final concentration of 65 mg/L. 16 Crayfish (8 males and 8 females; weight range: 14.6-19.7g) independent of all other study arms, were selected for use in the immersion study. 4 L of stock solution was added to six 113.6L totes (Bella, 30-gallon [120 quarts] Lowes, Mooresville, NC, USA) and crayfish were separated with porous plastic baskets (Y-Weave Cube Storage Basket, 2”X6”, Target, Minneapolis, MN), in the larger totes. This is the same setup as seen in **Fig. 4A**. Animals were immersed until an effect was seen, as described in the *Anesthesia Assessment* section, with a maximum trial period of 80 minutes.

19 Crayfish (10 males and 9 females), independent of all other study arms, were used in the Propofol injection study. Propofol, at a dosage of 100 mg/kg was injected into the VCHCZ, 1–2 cm off midline, using a sterile 1 ml syringe with a 25-gauge needle, ensuring to pull back on the plunger and visualize hemolymph in the needle hub to confirm placement into the open circulatory system **(Fig. 4B).** Following anesthesia trials of 65 mg/L propofol immersion and 100 mg/kg propofol injection; crayfish were placed in pre-conditioned facility water for recovery. Crayfish were assessed for the ability to recover from anesthesia over a 260 minute period (4.33h). Full recovery was considered an “awake crayfish” as described in the *Anesthesia Assessment* section.

Statistical Analysis.

The time of anesthetization and full recovery time between immersion and injection of propofol were tested for normality using the Shapiro-Wilk method. Due to failure of normality a Mann-Whitney Rank Sum Test was used to analyze by t-test

using the program SigmaPlot 14.0.0.124 (Systat software, San Jose, CA). The threshold for significance in all cases was $P \leq 0.01$.

Results

Life Support System and Water Quality Assessment. Parameters were collected daily over from day 0 to 15 and then on day 37 to quantify the environmental parameters in which *P. clarkii* were able to survive. The life support system and daily husbandry practices yielded a 0 % mortality rate during the entire study period. Laboratory water quality parameter values are summarized and included in Table 1. Quantitative statistical analysis of the water parameter data collected was performed showing the water conditions and normal variations over that time period. (Table 1)

Table 1
Water Quality parameters Mean values and standard deviations

	Mean	Standard Deviation
Crayfish	24	-
Temperature (°F)	71.71	± 0.80
pH	7.99	± 0.07
Conductivity (mS/cm)	336.53	± 22.29
Total Dissolved Solids (ppm)	239.37	± 15.93
Salinity (ppt)	0.17	± 0.01
Ammonia (ppm)	0.82	± 0.34
Nitrite (ppm)	2.27	± 0.22
Nitrate (ppm)	6.41	± 2.33
General Hardness (ppm)	158.56	± 7.94
Carbonate Hardness (ppm)	63.26	± 6.34

Health Assessments. None of the crayfish throughout the experiment were assigned a score of 0.75 for a single structure; as such a score was reserved for potentially life threatening injuries such as a cracked or crushed carapace. All crayfish in the experiment had a total score of 1.5 or less (maximal value 13), with 0.68 as the average damage score across the 24 crayfish in the housing experimental arm.

MS-222 immersion. No level of sedation or anesthesia of crayfish was observed throughout the 120 minutes of exposure at a concentration of 50 mg/L. No level of sedation or anesthesia of crayfish were observed throughout the 90 minutes of exposure at a concentration of 150 mg/L. All crayfish were determined to be fully awake. (Table 2)

Propofol immersion. All crayfish (n = 16) were sedated, as previously defined in the *Anesthesia Assessment* section, at a concentration of 65 mg/L Propofol. Sedation was seen in all crayfish. Time to effect was seen at 63.98 min (± 1.39 min) at 65 mg/L propofol. (Table 2)

Propofol injection. All crayfish (n = 16) were under surgical anesthetization, as previously defined in the *Anesthesia Assessment* section, when injected with 100 mg/kg propofol at the VCHCZ. Time to effect was seen in 16.63 seconds (± 16.38) at 100 mg/kg propofol. (Table 2)

Table 2
Propofol Immersion and Injection Temporal Anesthetic Parameters

	Time to effect (min)			Time to full recovery (min)			Mortality %
	Median	Mean	SD	Median	Mean	SD	
MS-222	N/A	N/A	N/A	N/A	N/A	N/A	0
Propofol Immersion	64.185	63.98	± 1.39	7.9	7.38	± 2.36	0
Propofol Injection	0.1667	0.277	± 0.27	139.31	164.5	± 77.28	15.8*

Time to effect was determined to be surgical anesthesia or sedation as described in the *Anesthesia Assessment* section. Time to full recovery considered an awake crayfish as described in the *Anesthesia Assessment* section.

*Mortality was due to investigator error in techniques, not the propofol injection. Please see discussion for further explanation.

Time to Effect. Comparison of Propofol administration techniques revealed a statistically significant difference in time to effect between the two routes. 100 mg/kg propofol injection provided significantly ($P < 0.001$) faster median time to effect compared with 65 mg/L propofol immersion **(Fig. 4)**.Injected *P. clarkii* median time to effect was 401 times faster than seen with immersed *P. clarkii* **(**Table 2**)**. MS-222 was excluded from these analyses since no observable effects were seen.

Recovery from anesthesia. Subjects fully recovered from propofol immersion in an average of 7.38 min (± 2.36) and propofol injection in an average of 164.5 min (± 77.29). Man-Whitney Rank Sum Test revealed statistically significant differences ($P < 0.001$) between routes of propofol administration. Visual assessment for adverse events and mortality were performed hourly, for the next 24 hours. No mortality was seen with propofol immersion and 15.8% mortality was seen with propofol injection **(**Table 2**).** Mortality was determined by lack of any movement for 24 hours, with no response to stimuli and lack of muscle tone.

Hemolymph Analysis. Direct slide preparations, stained with Wright Giemsa, of the crayfish hemolymph were minimally cellular, when compared to blood smears from vertebrate species, and had a moderately eosinophilic, proteinaceous background. Three different types of hemocytes were identified: hyaline, semi-granular and granular **(Fig. 5).**

The predominant hemocyte was the hyaline hemocyte. The hyaline hemocytes were round to occasionally spindle in shape and found individually. They measure 7–9µm in diameter and have a high N:C ratio with a scant amount of pale to moderately basophilic cytoplasm. The round to oval, eccentric nucleus has smooth to coarse chromatin with indistinct nucleoli **(Fig. 5A).**

The semi-granular hemocytes were round and found individually. They measure 10–13 µm in diameter and have a moderate N:C ratio with a moderate amount of pale basophilic, foamy cytoplasm that occasionally contain variably distinct, irregular slightly basophilic to eosinophilic granules. The round, central nucleus has smooth chromatin and indistinct nucleoli **(Fig. 5B).**

The granular hemocytes were round to oval and found individually. They measure 12–14µm in diameter and have a moderate N:C ratio with a moderate amount of clear to pale eosinophilic, foamy cytoplasm that often contains variable numbers of irregular purple to eosinophilic granules. The oval, occasionally round, often eccentric nucleus has smooth chromatin with indistinct nucleoli **(Fig. 5C).**

Discussion

To date there has been significant scientific publications that introduce methods on maintaining crayfish in a laboratory setting; However, there a far fewer studies that present life support systems in a biomedical vivarium. General crayfish life

support literature has specifically investigated best practices for stocking densities and basic environmental parameters to house crayfish (i.e., day:light photoperiod, ionized water calcium carbonate concentrations, and pH).[1,67,75,79,105,107,111,112] The authors' present a description of housing, husbandry, and selected clinical techniques in *P. clarkii* housed in a biomedical research setting, allowing for future evaluation of practices. The husbandry and housing described is economical, feasible, and reproducible in most laboratory animal facilities. Aquatic ecology experts were recruited to further investigate practical methods on assessing overall physical health, and behavior of the crayfish while housed in the life support systems. This setup provides a static system with individual tank aeration and basic bio-filtration, replicating the crayfish's natural environment, while in a vivarium. Commercial alternatives for bio-filtration and aeration are available throughout the industry if future investigators want to purchase pre-made foam filters instead of manufacturing them individually. The primary containment system ensured no crayfish escaped from their enclosures. This is especially important, as crayfish can and will escape if not properly housed. Researchers must be informed that currently there is no information available on reports of these specific clear polypropylene containers used in the study leaching toxic or immunosuppressive chemicals in the water over time. There were no adverse effects attributed to the containment system material during the duration of this study.

As explained in the introduction, this and many other species of crayfish is robust and thus can be invasive.[5,40,46,49,78,82,101] Prevention of non-native crayfish species entrance into local waterways is imperative during housing within a vivarium, as well as with disposal of animals. Before bringing crayfish into any research environment it's critical that investigators consult with local authorities and agencies who provide oversight for the movement of aquatic species (i.e., State and Country Departments of Agriculture, FDA). This is to ensure that if required the necessary permits and consent is obtained. Investigators must be aware that depending on the region requirements may vary.

Individually housed benthic animals have been shown to grow larger due to increased water volume available, and accurate control of individual environments.[26,61,75,93,105–107,113] Additionally, it is well known that *P. clarkii* are highly cannibalistic which is typically why individual housing is a necessity for this species.[6,26,45,56,71] For biomedical experimental trials, where reproducibility and proper biosecurity is paramount for validity, individual housing is imperative with novel specimens.[13,63]

Substrate would prove an optional aspect of the study. It allows for natural explorative behaviors as compared to a bare-bottom tank, since it simulates the natural environment. The use of substrate has been shown to promote increased survival rates in recirculating systems.[32,33] Investigators must be aware that substrate can be difficult to clean with a siphon, which could result in detritus material building up over time, altering water quality parameters.

All subjects displayed no adverse clinical signs throughout the entirety of the housing and husbandry study arm. A 50% water change every 7 days proved efficacious during the study period. In fact, normal molting behavior and egg production were seen in several of the crayfish, both species-specific behaviors supporting efficient replication of their natural environment (unpublished results). Future studies however, are required to further investigate the effects of the presented life support system on breeding, fecundity, and best stocking densities during longer experimental periods similar to Rodriguez-Serna et al. 2000.[79] Additionally, various levels of calcium carbonate in the water should be examined to determine optimal levels for molting, especially in young crayfish.[34,60,91] Although not investigated in this study, weak light intensities (38 lux) has been shown to improve the welfare of *Astacus astacus*, favoring exploration of lighted environments.[1]

Invertebrates are currently not covered under the Animal Welfare Act and many other countries currently exclude invertebrates in their regulations.[39,66,74] Many believe this stems from a lack of public concern for invertebrate welfare and the inability to conclusively show that distress occurs in invertebrates due to nociceptive stimuli. No matter what public opinion is, nociception has been seen in invertebrates and thus the welfare of these animals must be considered in the

laboratory.[24,35,80,83,109] Further reading is encouraged through Harvey-Clark (2011) for interested audiences.[35] During the early development of this study our institution's IACUC office was consulted. It was determined that a protocol was not warranted based on current regulations within the USA and to decrease regulatory burden. RSCHA used as a mechanism to quantify and assess welfare. Close collaboration was encouraged and maintained throughout the entire project to ensure that humane treatment was provided for these subjects. The institutions laboratory animal veterinarians were additionally consulted to provide guidance and oversight.

Although invertebrates are not considered to be a "covered" species in many of the animal research regulatory documents in the USA, authors urge that care must be taken when performing non-terminal hemolymph procedures.[74,96] (the guide, blue book, AWA) Current nonterminal bleeding standards in mammals limit to blood draws to 10% of total circulating volume, once every 3 to 4 weeks, if no replacement fluids are provided. Repeated blood samples cannot exceed 1% of the animal's blood volume, once every 24 hours.[72] Blood volume compared to weight is a negative relationship with increasing crayfish size, and inconsistencies have been seen when assessing carapace length to wet weight. Studies have shown that crayfish blood volume to weight ratio can range from 9–43%.[76,77] In *Limulus Polyphemus* 20–40% hemolymph collection has shown varying mortality from 0–30% depending upon the study.[7,41,54] We based our hemolymph collection limits and total blood volume off of mammalian studies, but further studies are required to evaluate the maximum total volume for nonterminal hemolymph collection in crayfish.[72,73] Of utmost importance while performing hemolymph collection (or giving injections through the abdominal segment) while at the VCHCZ, is that ventral midline is avoided. The ventral midline houses the ventral nerve cord, which is synonymous to the vertebral column of mammalian species.[8,78,83] The middle also houses the abdominal section of intestine which could cause bacterial translocation if aspirated. If the needle is inserted too cranial, the needle has the potential to enter the cephalothorax, and cause damage to internal organs.[27,38,44,82]

Authors chose VCHCZ site for hemolymph collection due to the restraint requirements of crayfish, allowing for clear visualization of anatomic landmarks. Previous works have noted collection of hemolymph from the ventral abdomen.[12]

Additional options for hemolymph collection are described in the literature for a number of different aquatic invertebrates. *P. clarkii* hemolymph collection can also be performed through pericardial cavity puncture, and inter-joint membranes.[16,69,86] Albores et al. (1993) have presented the following as a collection protocol: "The haemolymph was extracted from the pleopod base of the first abdominal segment near the genital pore" as an acceptable way to obtain 100uL of haemolymph in *Penaeus californiensis.*[99] *Shields (2017)* states that any crustacean with a >15mm carapace (width or length) can have hemolymph collection performed at the leg joint arthrodial membranes, or at the junction of the abdomen and carapace with a 27 gauge needle.[81] In *Litopenaeus vannamei*, hemolymph can be collected with a 26 gauge needle from the first abdominal segment venous sinus.[85] In *Limulus Polyphemus*, blood collection is typically from the heart itself at the joint where the prosoma and opisthosoma meet.[11] The varied collection techniques for hemolymph have not been investigated formally in a study for efficacy or translational ability, and would provide useful information on guidance for best practices.

Commercially available alternatives, that include sodium thiosulfate, are also appropriate for dechlorination of water from laboratory sources. Typically these solutions are around 3.175g/L Sodium thiosulfate.[68,98]

MS-222 is currently the only FDA approved drug for use in anesthesia in fish and other cold-blooded aquatic animals, and thus commonly used, indicating the need for further investigation in crayfish.[9,25,95]Immersion of MS-222 did not have any anesthetic effects on the crayfish used in this study. This is in agreement with many published works.[17,23,88] Further investigation with immersion at higher doses or direct injection into the hemolymph may be warranted. Propofol was utilized for sedation methods within this study, as it has been shown to be safe and have neuro-protective effects.[2,3,48,52,93] Propofol is well researched throughout the literature.[18,31,52,59,64,97] Propofol must be used under veterinary supervision, but

it is not a controlled drug under DEA guidance.[4] This makes propofol easy obtained in a biomedical environment that has laboratory animal services department, as veterinary staff is familiar with the handling, use, and disposal of this commonly used and inexpensive drug. Other options for sedation including clove oil, cooling, $MgCl_2$, and CO_2 were not investigated in this study.[28,30]

Although mortality rate observed with injection was 15.8%, this was due to investigator specific errors in three of the tested crayfish. One crayfish had a handling induced thorax fracture in association with recent ecdysis. Two other crayfish experienced multiple traumatic needle insertions during propofol administration into musculature and nerve cord due to improper injection technique. Thus avoiding these mistakes is imperative for the appropriate collection of hemolymph. Future studies on VCHCZ utilization would be useful for optimizing this approach. Propofol injected into coelomic hemolymph provided effective anesthesia for non-invasive or experimental techniques and research procedures, with the longest duration of action at 100 mg/kg when compared to propofol immersion. With an anesthetic effect lasting 164 minutes, investigators would have sufficient time to perform procedures that may require lengthier anesthesia periods for experiments (i.e., imaging modalities: PET-CT, ultrasonography). This duration of time also creates a long post-operative period, which could be inappropriate for some study protocols. Future studies are indicated to determine if this dose is appropriate for surgical interventions and if the dose could be optimized for more succinct time management. Propofol immersion provided enough sedation, or light anesthetization, for safe and effective handling. Immersion could be utilized in preventing injury to the crayfish and the investigators during personnel training sessions. Future work is needed to optimize the propofol concentrations and dosing.

Our team was able to successfully demonstrate effective techniques on obtaining for cytological evaluation without any animal losses. It is important to note that authors recommend analyzing fresh collected samples within a 2-hour period using EDTA tubes. Previous works have demonstrated the effectiveness of EDTA solutions for hemolymph storage, and adaptions of protocols were used in this experiment.[12,47,65,99]

In summary authors also present practices and data that could be incorporated when housing other crayfish in the genus *Procambarus* and other related species used not only in biomedical research, but also in aquaculture settings if warranted.

Declarations

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Figures

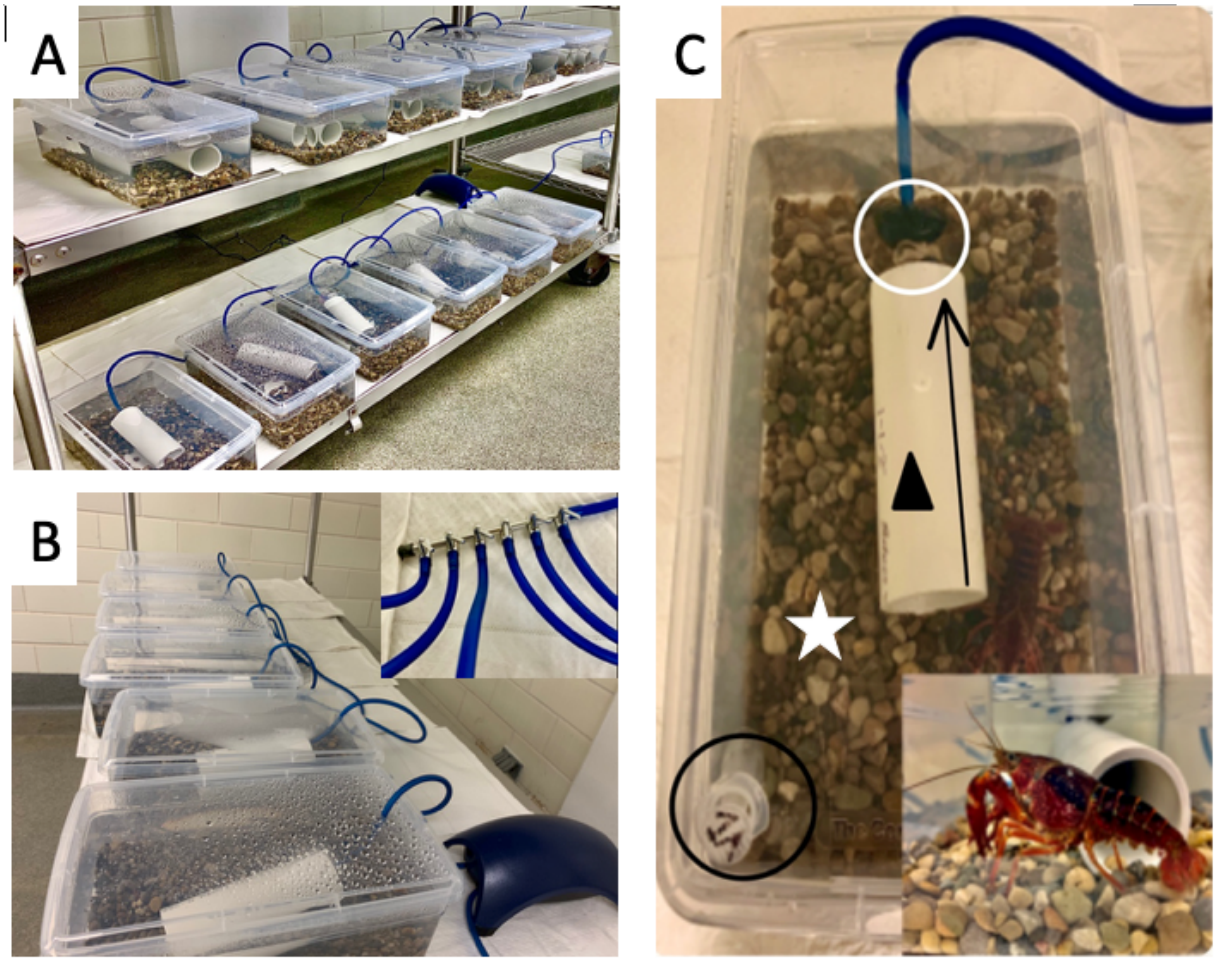

Figure 1

Images of crayfish life support system. (A) Animal racks containing individually-housed Procambarus clarkii. (B) Air source (□) with close up of Six-Way Air Flow Lever Valve in inset (C) Primary Containment System. □(white)□Pea gravel substrate, Δ Stress reduction hide, O(black) water collection syringe, O(white) Foam biological filter and ⬆ direction of water flow through filter . Procambarus clarkii used in experiment in inset.

Red Swamp Crayfish Health Assessment Scale

CRAYFISH ID: 1A	Damage level					Note:
Anatomic Part	Absent Part (1.00)	Major (0.75)	Medium (0.50)	Minor (0.25)	None (0.00)	
Left Cheliped						
Right Cheliped						
Antennae						
Antennules						
Maxiliped						
Carapace						
Abdomen						
Telson						Total Score:
Uropod						
Left Eye						
Right Eye						
Rostrum						Out of 13
Walking legs						

Figure 2

Red Swamp Crayfish Health Assessment Scale

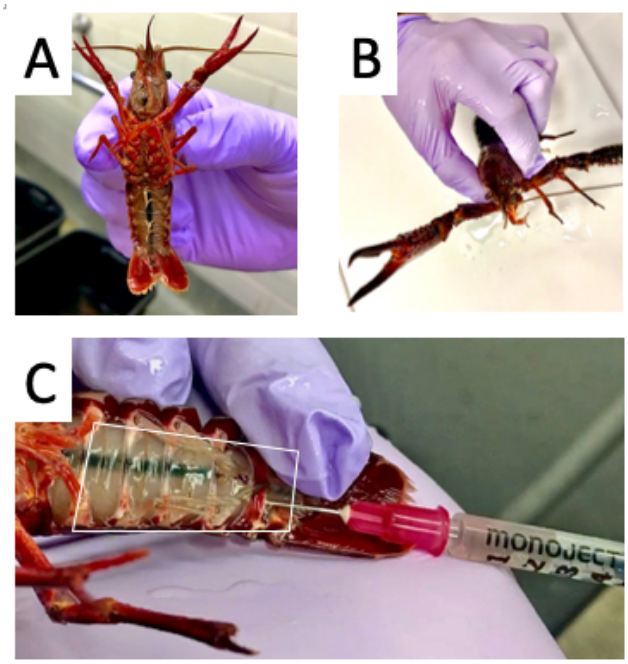

Figure 3

(A & B) Image demonstrating proper handling and restraint. (C) Technique for hemolymph collection as described in Restraint and Hemolymph Collection at the Ventral Ceolomic Hemolymph Collection Zone (▯ white).

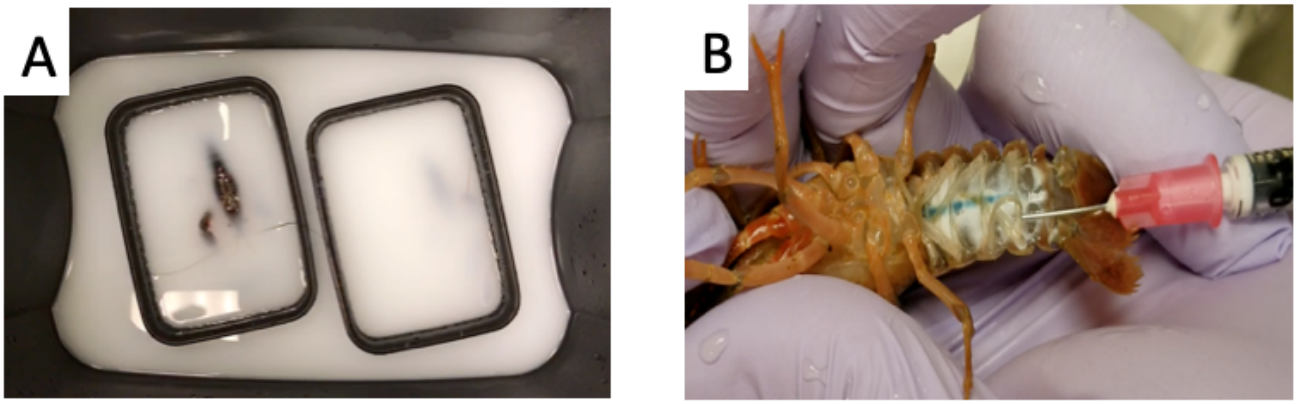

Figure 4

Propofol routes of administration. (A) Image of two Procambarus clarkii immersed in 65 mg/L of propofol and conditioned water. (B) Image demonstrating proper administration of Propofol through intracoelomic route of administration at 100mg/kg. Note: syringe is offset from midline, away from the nerve cord(⬆). Propofol is being injected posterior to the sternum of the 5th abdominal somite, lateral to midline to avoid the nerve cord within the VCHCZ (▯ white).

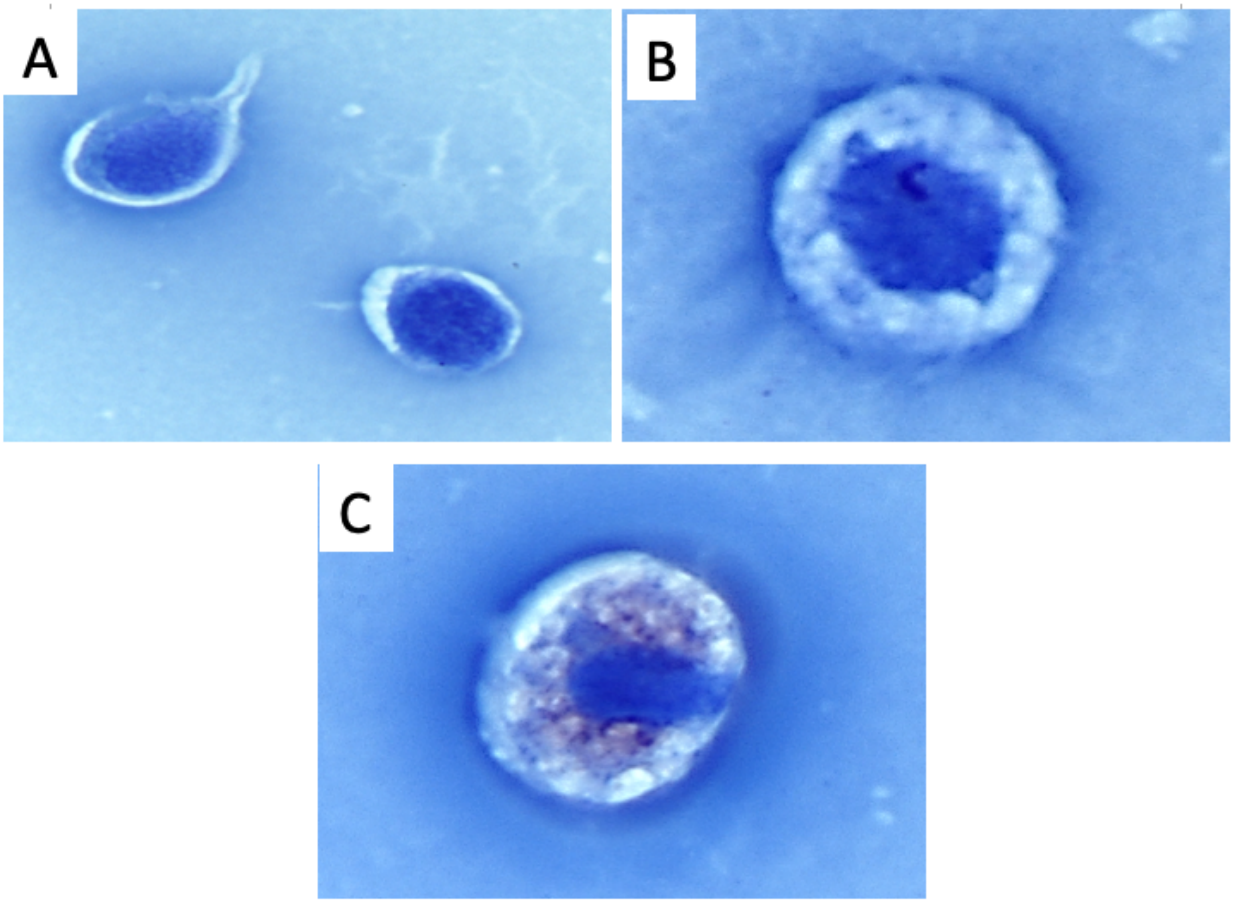

Figure 5

Wright. Cytological Evaluation of Hemocytes with Wright Giemsa stains for Normal Hemocyte populations.(x100) (A) Hyaline Hemocytes (B) Semi-Granular Hemocytes (C) Granular Hemocytes

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- sedationexp.pdf
- WaterCollectionData.pdf
- sedationexp.xlsx
- WaterCollectionData.xlsx