IACUC Approval Section 3

Snapping turtles, *Chelydra serpentina*, have long been thought to be a fairly quiet organism, but more recent studies have proven that these intriguing animals have the ability to vocalize through various chirps, clucks, hisses, and clicks. Although the amount of research has been increasing with C. serpentina vocalizations, very little is known about the vocalizations of snapping turtle hatchlings and the role that vocalization plays in turtle behavior. Further studies on this topic, which include audio recordings and autonomous underwater vehicle (AUV) tracking, can help to better understand the dispersal patterns of turtle hatchlings, the interactions between turtles, and other behavioral aspects that may be affected by vocalizations. The use of AUV's is imperative to this research in order to record and analyze the vocalizations. C. serpentina make good model organisms as they are not the fastest species on land or water by any means, so they are easy to catch. Furthermore, they lay 25-80 eggs each year, providing large generations to study, and they provide a large body surface area so that trackers or tags can easily be placed. Additionally, the furthest a snapping turtle will travel from its pond is around 10 miles, so they are not extremely difficult to track if they do shift homes.

1. Navigate an AUV towards turtle species by detecting chemical secretion gradients in a local body of water.
    a. Hypothesis: The presence of chemical signal components such as cholesterol trimethylsilyl ether - specific to freshwater turtles - can be detected in a gradient via a pair of electrodes to provide heading information.
    b. The most commonly used olfactory-based navigation algorithm is "chemotaxis" , which was introduced by Berg and Brown (Berg & Brown , 1972; Berg, 1993). This strategy is based on the detection of a concentration difference between two chemical sensors and a steering mechanism (microcontroller, data processing, and motors in this case) toward the direction of higher concentration with a constant moving speed. Polarographic electrodes mounted on the sides of the AUV comprise the physical design. The majority of the directional determination and quantification of chemical signal concentration is done with software algorithmically (Farrell et al., 2005). Sensors with more extensive capabilities than conductance, e.g. mass spectrometers, Raman spectrometers, and fluorometers are too bulky and massive for this study. After calibration of resistance to known freshwater turtle chemical signals such as trimethylsilyl ether, a gradient can be established between the two electrodes. I.e., electrode 1 appropriate resistance signal > electrode 2 signal == gradient differential. Resistance values will likely be on the order of milliohms (mΩ). Chemical signals released by C. serpentina or other turtle species may be detectable and navigable with

an AUV. The most common compound is likely cholesterol trimethylsilyl ether or a derivative (Ibáñez et al. 2020). Based on the electrical conductivity, the presence of a signal in the area may be detected. A chemical gradient is key for navigation, additionally the data will still provide knowledge of chemical signal presence but without a heading.

2. Audio recordings allow for specific detection and understanding of C. serpentina vocalizations. We will take audio recordings in-situ. The audio can be analyzed with a spectrum analysis to isolate Chelonia-like sounds. Isolated sounds can then be compared with known C. serpentina audio for distinct features.
    a. Hypothesis: The recorded audio can be parsed despite background noise to reveal vocalizations specific to C. serpentina outside of captivity.
    b. While there have been studies estimating the frequency range of *C. serpentina* vocalizations, these vocalizations are generally from adult turtles and are not yet well understood, especially outside of captivity. Vocalizations of *C. serpentina* populations in captivity have been studied a little more, but these recordings are isolated from the noise pollution naturally found in underwater habitats and the turtles themselves may behave differently under laboratory settings vs in its natural habitat. By recording the vocalizations of *C. serpentina* hatchlings in their natural habitat, we will produce more accurate information on their natural vocalizations and frequency range. We will also be developing methods to isolate *C. serpentina* hatchling vocalizations from the audio recorded by the hydrophone, thereby providing future researchers with a better foundation for studying not only snapping turtle vocalizations, but perhaps more generally underwater vocalizations in non-captive environments. Previous studies, such as Ferrara C., et al (2012), focusing on snapping turtle vocalizations have recorded turtles both in and out of captivity, but primarily focused on adult turtle vocalizations. For these reasons, it is imperative that we work with wild populations of *C. serpentina* hatchlings for this study, as there has been little research into the vocalizations of these creatures outside of captivity and there is no suitable substitute to study their vocalizations from.

3. This is important to test the effectiveness of AUVs for future studies. Some C. serpentina vocalizations are too low for the human ear which is why using effective equipment is

important. It is thought that C. serpentina hatchlings produce different sounds before they are hatched. The vocalizations must be recorded post hatching to observe differences in pre hatching vocalizations.

a. Hypothesis: Pre-hatchling vocalizations will be 20-30 Hz lower in range than post-hatchling vocalizations.
b. While previous studies have been done regarding turtle vocalizations, very few have been focused on *C. serpentina* hatchlings. A research study from the University of Toronto involved recording *C. serpentina* hatchling emergence in a controlled captive environment (Lacroix et al., 2020). The results of this study concluded that vocalizations increased in frequency and complexity during the later stages of hatchling emergence, specifically directly before and during emergence. It was also stated that hatchlings emerging in groups proved to be energetically favorable. The study did not continue to record *C. serpentina* hatchling vocalizations after emerging from their eggs. The lack of recordings and information available concerning *C. serpentina* hatchlings post-emergence and in their natural environment is where our research can be beneficial.

IACUC Approval Section 4

Summarize sequentially <u>all</u> animal procedures:

The wild snapping turtles will be studied at two pond locations within the New Munster Wildlife Area and will remain in this area throughout the study for all specific aims. The New Munster Wildlife Area was selected because open wetlands constitute 25% of its total area, providing reed grasses in which the turtles can seek shelter or refuge. The oak and lowland woodlands, shallow marshes, open grassland, and agricultural fields in the area would also provide turtles with a relatively contiguous habitat, and the nearby Palmer and New Munster Creeks flowing into the Fox River would provide ample food. The specific pond sites we will focus on are isolated enough to provide a safe space for research but close enough to roads or trails that they would not be too difficult to get to. There are also canoe launching sites if shoreline vegetation makes launching the AUV too difficult to do from land. While 12% of New Munster Wildlife Area's cover is forested wetland, the ponds we have selected appear to be relatively open, allowing for easy movement of the AUV within the aquatic environment. During the summer months, the DNR reports these locations to have a high water clarity, making visual navigation of the AUV easier.

On foot, we will spot and track gravid Chelydra serpentina females back to their nest. The females will have their date of oviposition recorded, and the nest site locations will be recorded and protected with a wire-mesh cage to reduce the risk of terrestrial predation. Additionally, these sites will

be physically marked with a red flag about 5-6 feet away. Following the date of oviposition for each gravid female, we should be able to collect the hatchlings as soon as they emerge from their eggs. At this point, the hatchling turtles will be brought to the mobile lab for assessment, tagging, and release. Medical procedures such as euthanasia or anesthetization will not be performed on any of the turtles during this study.

Each hatchling will have their shell size measured and body weight recorded to ensure they meet the minimum requirements of 50mm in carapace length, or 7g in total weight. They will also be inspected for any signs of injury, deformity, or disease, as we only want to use healthy hatchlings in this study. Turtles with signs of recent injuries, or that appear ill will not be tagged. The PIT tags we will be using are Biomark's MiniHPT8 RFID tags, preloaded into 16 gauge needles. The complimentary MK65 implant gun will be used to inject the hatchlings with the RFID tags. Most veterinarians do not consider PIT tagging to result in an increased health risk for the hatchling turtles if the tags do not enter the body cavity. Under sterile conditions, and using aseptic technique, injection of the RFID tag will occur subcutaneously in the left hind limb of the hatchlings, parallel to the femur. When done correctly, there should be little, if any, bleeding post injection. If bleeding does occur, a small amount of pressure will be applied with a sterile gauze or cotton ball covering the wound until bleeding has stopped. After injection, the wound will be treated using a liquid bandage antiseptic solution to help prevent infection. Each hatchling turtle will then be observed a minimum of 20 minutes and a maximum of 24 hours to ensure their health before release. Healthy hatchling turtles eligible for release will be scanned with the PIT tag reader, before being released back into their appropriate nesting sites.

Aim 1. AUV chemotaxis navigation requires only measurements of the surrounding water to detect turtle chemical secretions and utilize them for navigation. Polarographic electrodes mounted on the AUV will comprise the physical design. A microcontroller and onboard data processing will be used to extrapolate turtle chemical directional and presence information; directional information is dependent on the existence of a chemical gradient in the local environment. The majority of the directional determination and quantification of chemical signal concentration will be done with software algorithmically. After calibration of resistance to known freshwater turtle chemical signals such as trimethylsilyl ether, a gradient may be established between the two electrodes with resistance values. The best algorithm based on a summary of chemotaxis algorithm strategies by Farrell et al. 2005 will be applied experimentally to the data to extrapolate a heading where the chemical signal plume is likely coming from. The heading information will then be applied to the AUV thrust. Due to the nature of this chemotaxis method of navigation, little to no contact with snapping turtle hatchlings is ever required.

Aim 2 and 3. Vocalizations of the hatchlings and adults will be recorded using a Cetacean Research Technology SQ26-H1 Portable Underwater Sound Recording System. The hydrophone will be positioned 0.5 meters from the bottom and its sensitivity will be set to 1.5Hz-10kHz ± 3 dB and

sampled at 22kHz for hatchlings and adults. Recording period will occur for two periods of 4 hours each day from 8:00AM to 12:00PM and 4:00PM to 8:00PM. Recording months will go from April through October as this is the mating and nesting season for C. serpentina. Hatchlings will begin to emerge in August. While recording, we will monitor the audio in real time to account for any outside disturbances or airborne sounds and adjust the signal to noise ratio to prevent audio distortions and isolate the turtle vocalizations.

Animal identification techniques :

- Microchip

Age of animal at time of identification procedure:

- Hatchlings

Anesthesia used for identification procedure:

- N/A

Provide specific agents, doses, and route of administration for euthanasia of each species:

- N/A

If requesting either cervical dislocation or decapitation without anesthesia as a method of euthanasia, provide scientific justification:

- N/A

For all procedures, indicate which have not been performed in this laboratory before and how personnel will be trained in new techniques?

Tagging procedures have not been performed in this laboratory before. Personnel will be trained in PIT tagging hatchling turtles based on techniques and methods of previous studies involving hatchling turtles, as well as instructional videos on how to use the Biomark MiniHPT8 RFID tags and the complimentary MK65 16 gauge injection gun.

Is there a potential for the development of morbidity? (Note: Procedures such as surgery, tumor induction, transgenic experiments, injections of adjuvant, prolonged restraint, water restriction, ascites production, etc., all have inherent risk of medical problems).

Most veterinarians agree that subcutaneous injection of RFID tags in the hind legs of hatchling turtles do not pose an increased risk to the hatchling's health. A small amount of bleeding may occur, but morbidity is unlikely to occur from this procedure.

Will <u>all</u> animal care and use be conducted on-campus

No, all work will be done in-situ at a mobile lab in the New Munster Wildlife Area. Pond A is located at 42°34'51.1"N 88·12'42.6"W, and Pond B is located at 42°34'42.81"N 88°12'30.08"W. At these locations, we will be tracking the hatchlings using chemosensory navigation in the AUV and recording their vocalizations. Animal care (during the tagging procedure) will be performed in the mobile lab on site.

Will you remove living animals from animal facilities or bring them into the lab from off-campus?

No, all animal care will be done in-situ.

***Describe how animals will be transported to or from areas outside of Carthage animal facilities, and whether they will be returned afterwards to Carthage animal housing; specify Carthage rooms in which animals are housed (if known):**

N/A

Section 6:

Describe study group sizes and number of experiments in the next year that justify the animal numbers requested under Categories B, C, D and E. If breeding animals in-house, be sure to account for numbers needed to maintain the colony. Include evidence of retrospective or prospective statistical methods that support your estimates.

The number of snapping turtles that will be examined and subjected to category 'C' strains is estimated to be no more than 50. This number is based on the average clutch sizes of C. serpentina. It is necessary to tag as many nest sites as possible and as many individual turtles as possible, as various predators will likely be present in the area including the largemouth bass, panfish, northern pike, and the great blue heron. By attempting to use as many available individuals as possible in the study, it will allow for a more complete data set in the end that may be more accurately applied to larger populations of snapping turtles. The strains encountered by snapping turtles in this study are a result of the PIT tagging process required for individual identification. In order to have complete and consistent data, knowledge of the individual's history in relation to this study is required.

When performing the study in the New Munster Wildlife Area, it is estimated that 1 - 3 nesting sites will be found within the specific area of interest that operations will take place. It is these nests that will have provided the hatchling individuals for tagging and vocal recording. Unfortunately, the ecology of hatchling turtles is poorly known, despite its importance to management plans and to life-history models (Morafka 1994). This is mainly because turtle hatchlings often occur in low densities, are relatively inconspicuous and enjoy dense natural cover (Graves & Anderson 1987), and until recently, radio-tracking and identification technology was too large for use on such small animals (i.e., <6 g).

Animal use since last annual renewal: Using a retrospective evaluation of expected and unexpected outcomes, how many animals of each species have been used since the last protocol review under Categories B, C, D and E?

- N/A