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Standardizing a Non-Lethal Method for Characterizing the Reproductive Status and Larval Development of Freshwater Mussels (Bivalvia: Unionida)

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TITLE:

Standardizing a Non-Lethal Method for Characterizing the Reproductive Status and Larval Development of Freshwater Mussels (Bivalvia: Unionida)

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KEYWORDS:

imperiled species, endangered species, conservation, life history, glochidia, gravid, reproduction

SUMMARY:

Freshwater mussel conservation is dependent on monitoring reproductive patterns and processes of species. This study standardizes a non-lethal protocol for sampling gill contents, characterizing larval development, and providing a digital repository for data collected. This protocol-database package will be an important tool for mussel researchers in recovery of imperiled species.

ABSTRACT:

Actively monitoring the timing, development, and reproductive patterns of endangered species is critical when managing for population recovery. Freshwater mussels are among the most imperiled organisms in the world, but information about early larval (glochidial) development and brooding periods is still lacking for many species. Previous studies have focused on the complex life history stage when female mussels are ready to parasitize host fish, but few studies have focused on the brooding period and timing of larval development. The protocol described here allows researchers to non-lethally evaluate the state of gravidity for female mussels. The results of this study show that this method does not affect a female mussel's ability to stay gravid or become gravid again after sampling has been performed. The advantage of this method may permit its use on federally threatened or endangered species or other populations of high conservation concern. This protocol can be adapted for use on both preserved or live individuals and was tested on a variety of mussel species. The database provided is a repository for a breadth of information on timing of reproductive habits and will facilitate future freshwater mussel research, conservation, and recovery efforts.

INTRODUCTION:

The persistence of populations in freshwater systems depends on the success of reproduction and recruitment. For parasitic organisms, identifying the intricacies of the life cycle (e.g., stages of larval development and host attraction strategies) can give insight into an organism's reproductive habits and critical processes that influence recruitment. Such information becomes important when species are imperiled, and successful recruitment is needed to sustain remaining populations, or if recovery necessitates the use of captive propagation for reestablishing extirpated populations.

Freshwater mussels (Bivalvia: Unionida) are considered one of the most imperiled groups of organisms worldwide and a compilation of species-specific reproductive habits could aid in research efforts[1-5]. With over 800 currently recognized species distributed across the globe, freshwater mussels have hotspots of diversity in North and South America, and southeastern Asia but essential life history information is unknown for many species[2,5-7]. Families within this order are characterized by having parasitic larval stages that complete metamorphosis into free-living juveniles during attachment to a host[7,8]. This unique life history stage contributes to the biodiversity in freshwater systems, which are currently in crisis[9]. High levels of imperilment can be attributed to many anthropogenic threats including pollution of waterways, habitat alteration and destruction, reductions in the abundance and diversity of host fishes, and introduction of invasive species[1,10]. As benthic filter feeders, mussels burrow into the substrate and are susceptible to contaminants and pollutants that drain into the watershed[11]. Recovery of mussel species is pertinent as they provide a wide variety of ecosystem services, including carbon sequestration, a food source, and water purification by filter feeding[11]. In addition, mussels have been found to indicate ecosystem health, promote biodiversity, and in turn, increase resiliency of an ecosystem[12].

Many freshwater mussel studies have focused on investigating early life history requirements to better inform species status assessments and management strategies. The freshwater mussel families relevant to this study (e.g., Hyriidae, Margaritiferidae, Unionidae) have a unique life history strategy where females brood larvae (glochidia) in their marsupial gills[8]. Through a variety of strategies, the female mussel expels mature glochidia from marsupial gills to parasitize a vertebrate host with glochidia[13]. Research on glochidial development within the gills was modified from a technique utilizing hypodermic syringes to sample gonadal fluid from live mussels and evaluate gamete production[14-16]. As researchers validated this non-lethal methodology for gonad sampling, it was adapted for marsupial gill sampling to evaluate brood development[15,16]. Brood development can be used to decipher phylogenetic relationships as some mussel species can brood glochidia in only the outer two gills (ectobranchus), only the inner two gills (endobranchus), or in all four gills (tetrabranchus) but this characteristic is not known for every species[17]. Brooding patterns have previously been used to classify mussel species by whether female mussels brood glochidia over winter (bradytictic) or for a short period in the summer (tachytictic)[18]. The over wintering of mussel broods was supported when the reproductive cycle of *Anodonta* was studied[19]. However, basic reproductive biology was studied more thoroughly over the years and found this dichotomy was a gross generalization and brooding periods of some species are much more complex than originally presumed[20,21]. For

example, species of the genus *Hyridella* (family Hyriidae), *Glebula,* and *Elliptio* (family Unionidae) have been observed with upwards of three broods per breeding season[22-24]. The complexity of species-specific, and sometimes even population-specific[20], reproductive habits has led to a gap in knowledge about the timing and duration of brooding, and number of broods a female mussel may produce.

Although hypodermic syringes have been used to extract gill contents, reporting the results is complicated due to the lack of standardization to ensure comparable results across all studies. Previously, four developmental stages of glochidia (i.e., egg, embryo, immature, fully developed) have been identified in Unionidae but have not been adopted into standard procedure[16,25,26]. Other studies observing members of Margaritiferidae have substituted the classification of 'immature glochidia' with 'developing glochidia', leading to potential confusion[27,28]. The lack of consistency in characterizing the different larval development stages has left many researchers to generally describe brooding females as 'gravid', which does not encompass the intricacies of larval development. Life history studies conducting host-fish trials have prioritized the need for gravid females with fully-developed glochidia, but this information is scattered throughout published and unpublished literature[29,30]. Currently, data are lacking about the reproductive habits of many mussel species, including timing of the transition between egg, immature glochidia, and fully developed glochidia ready for attachment to hosts. For most species, it is unclear how long females brood glochidia and how quickly fertilized eggs fully develop. The knowledge gaps are often wider for species of conservation concern, which presents the need for a standardized method of extracting gill contents that has been tested for non-lethal effects and can be promoted to the scientific community to supplement mainstream data collection methodologies, without posing a threat to protected populations[24,31,32].

This study had three objectives: 1) formalize a gill sampling technique and test it for lethal and non-lethal effects on female mussels in situ, 2) characterize different stages of glochidial development and describe a standardized method of identifying and reporting various larval stages, and 3) create a public repository for the data collected. Field surveys, long-term monitoring projects, and museum collections all represent opportunities for the protocol described here to be implemented and additional data to be collected for a wider body of interest. The formalized protocol includes visuals and character descriptions for differentiating each stage of larval development. By standardizing the categories, the results collected can be compared among all occurrences and species. Once data are collected, all can be submitted to the Freshwater Mussel Gravidity Almanac (FMGA), which is a database for gravidity information collected using this protocol. A final product to store and compile all the gravidity information collected will provide a research tool to facilitate future research, conservation, and recovery efforts. The incorporation of this methodology into various mussel projects and the submission of data to FMGA would expand upon the breadth of knowledge concerning gravidity status of mussel species throughout the year. As a highly imperiled group of organisms, this protocol and resulting database on the reproductive habits of freshwater mussels is essential to understanding population dynamics and facilitating the conservation of these species.

PROTOCOL:

1. Gravid female collection

NOTE: Reference the Fish and Wildlife Service's Freshwater Mussel Survey Protocol[33] for guidance on how to adequately survey a sampling site for threatened or endangered species. Proper federal permits must be obtained before field collection of protected species and state permits for all species present.

1.1. Collect live mussels from the field using tactile-visual methods (step 1.2) or utilize preserved specimens from a museum (step 1.3).

NOTE: It is important to keep live mussels cool and wet after collection to prevent desiccation and reduce stress, and minimal handling of gravid mussels is important to avoid females prematurely releasing gill contents[34].

1.2. Evaluate female gravidity either by visual inspection during collection (e.g., presence of mantle lure, conglutinates, etc.) or by visual inspection after collection (e.g., gently prying open valves enough to look inside and see whether gills are inflated, see **Figure 1**).

NOTE: Species vary in how glochidia are brooded within the marsupial gills as sometimes only the two outer gills (ectobranchus), only the two inner gills (endobranchus), or all four gills (tetragenous) are marsupial[17]. The protocol can be paused here, and gravid females can be transported back to the lab for gill sampling.

[**Figure 1** shown here]

1.3. Perform a visual inspection on preserved specimens by opening the valves and inspecting the gills to determine whether the individual is a gravid female (**Figure 2**).

[**Figure 2** shown here]

2. Gill content sampling

NOTE: This protocol can be adapted whether sampling occurs on live mussels in the field and laboratory, or on preserved specimens.

2.1. Prepare a 1.5 mL plastic microcentrifuge collection tube with approximately 1 mL of either sterile water if gill contents will be evaluated within 24 h of extraction[35] or ethanol (EtOH) if sample evaluation cannot occur within 24 h of collection or if gill contents are from a museum specimen preserved in EtOH. If glochidia are intended for scanning electron microscope (SEM) imaging, use 70% EtOH, and if glochidia will be used for genetic testing, use non-denatured 95% EtOH[36].

2.2. Remove the paper wrapping for one sterile 20 G bevel-tip needle on a 10 mL syringe.

Unscrew the cap to expose the needle and prepare a 1.5 mL plastic tube for gill content collection. Push the handle of the syringe all the way down so the black stopper is at the 0 mL/cc line.

NOTE: A sterile syringe should be used each time gill contents are sampled. A used syringe can be sterilized in the field by dipping the tip in a 10% bleach solution, then rinsing the syringe by filling it with 1 mL of sterile water and depressing the plunger back to 0 mL/cc, and finally drying the syringe with a clean cloth.

2.3. Pick up the gravid female and gently pry open the two valves using the tips of the thumbs.

CAUTION: Be careful to not harm the animal. Opening the valves too wide or too fast can overextend adductor muscles and cause mortality. Thin-shelled specimens (e.g., species of *Anodonta*, *Leptodea*, *Utterbackia*, etc.) and young individuals are especially vulnerable in this step. Forcefully handling fragile-shelled species may crack the shells and cause mortality. In some cases, squeezing thin-shelled animals from the anterior and posterior shell margins, while looking at the ventral surface, will cause the shell to flex and gape slightly, allowing one to observe the gills or pry open the shells and avoid damaging the fragile shell margin.

NOTE: Tools can be used to assist with this step but can also cause mortality if not used with care and should be avoided whenever possible. For example, a speculum or modified set of reverse pliers may be used to help pry the individual open and a wedge can be used to help prop the valves open. These instruments may not be necessary if another person is available to assist (i.e., one person holds the animal open while another maneuvers the syringe for extraction). Damaging or separating the mantle tissue from the periostracum can cause growth deformities and mortality[37]; therefore, it is critical to avoid severing the connection between the mantle tissue and outside margin of the shell.

2.4. Use the needle tip of the syringe to gently penetrate a single water tube of the inflated marsupial gill. Next, gently scoop the gill contents out by utilizing the beveled tip of the needle.

NOTE: Gill contents usually have a milky-white consistency, which should be visible on the beveled tip of the needle.

2.4.1. Deposit the contents of the syringe directly into a Petri dish if a microscope is readily available. Otherwise, store the contents in a 1.5 mL plastic microcentrifuge tube with designated liquid (see step 2.1) for later evaluation.

NOTE: Minimize disturbance and handling of glochidia samples during transportation to avoid damage and reduced viability[32,35].

2.5. Record information on the genus-species identification, gravidity status, length of female (mm), collector and contact information, state, county, drainage, specific collection location, latitude and longitude, a unique identifier for the gill sample, a unique identifier for the survey site, and the date of collection if gill contents were extracted (**Figure 3**). Record a unique identifier

on each collection vessel to ensure accurate data records during transport.

2.5.1. Photograph the outside right valve of the mussel for identity validation and include the tube labeled with the unique identifier legible in the picture. Optionally, collect other abiotic and biotic parameters to supplement the information on the environment and community the mussel was found in (see **Figure 3** for suggestions).

[**Figure 3** shown here]

3. Laboratory evaluation of gill contents

3.1. If gill contents are in a 1.5 mL tube, transfer them into a Petri dish and fill the bottom of the dish with water. Gently swirl the Petri dish in a circular motion to collect contents in the center of the dish for a more concentrated view of the sample.

NOTE: The 1.5 mL tube may need to be flushed out using a squirt bottle or transfer pipet filled with water if gill contents are sticking to tube walls.

3.2. Place the Petri dish under a dissecting microscope to evaluate the sample. If possible, take a photograph of the gill sample under the microscope and label it with the unique identifier for that sample.

3.2.1. Record results of which developmental stages are present in each gill sample. Use **Figure 4** as a guide to characterize each developmental stage. In some cases, females may be brooding larvae at multiple developmental stages; therefore, report every developmental stage observed within a given sample (e.g., 'EGG/DG/IMG/FDG'). Once preserved glochidia have been evaluated, proceed to section 4. If fully developed glochidia are identified and EtOH was not used for preservation, proceed to step 3.3.

NOTE: EGG, egg masses; DG, developing glochidia; IMG, immature glochidia; FDG, fully developed glochidia.

[**Figure 4** shown here]

3.3. Conduct a sodium chloride (NaCl) test to further evaluate viability of any fully developed glochidia by adding a crystal of NaCl to a subset droplet of the gill sample[35]. Viable glochidia will respond to NaCl by opening and closing their valves or simply just closing from an open position. Report any salt-tested glochidia with '(T)' at the end of the designation when data are recorded.

NOTE: Fully developed glochidia may also be observed actively snapping open and closed without exposure to NaCl.

4. Report to database

4.1. Access the FMGA web (http://arcg.is/089uee), which was developed using online software programs[38-40]. The FMGA page provides a link to the desktop data entry form and an application download for mobile devices. The mobile app enables data entry in the field and automated georeferencing[41].

NOTE: The gravidity calendar and other graphics associated with freshwater mussel species life history events can also be found on the FMGA Dashboard.

4.1.1. Use the mobile app or the desktop site to record results in the data entry form by utilizing drop-down menus and text entry fields. For large, pre-existing datasets, download a template spreadsheet on the desktop site. Enter recorded data under the appropriate column headings, keeping in mind that each record, or row on the spreadsheet, represents observations of a gill sample from one gravid individual.

4.1.2. Submit the results and they will be added to the FMGA database after being validated by an administrator, who may contact the collector to request further details or photos.

NOTE: Once data are validated and compiled in the FMGA database, all gravidity calendars and other interactive graphics displayed on the FMGA dashboard will be updated.

REPRESENTATIVE RESULTS:

This protocol was applied during a capture-mark-recapture study that monitored the freshwater mussel community within a 750 m^2 stretch of Bruce Creek (Walton County, Florida) from January 2015 to December 2015. Field sampling was scheduled to occur every four weeks; however, due to high-flow events, sampling was not conducted in April or September of 2015. State and federal agencies including U.S. Geological Survey, U.S. Fish and Wildlife Service, and Florida Fish and Wildlife Conservation Commission assisted in field surveys and gill sampling. Each gravid female encountered during the survey was subjected to in-field gill content sampling using the protocol described above, tagged (see **Table of Materials**), and placed back into the river substrate. The gill samples were stored in 95% EtOH and transported to the U.S. Geological Survey's Wetland and Aquatic Research Center's laboratory for evaluation of gill contents.

By tagging females and recapturing them at monthly intervals throughout the year, we evaluated both lethal and non-lethal impacts of the gill sampling protocol on a total of 90 individuals. The following seven species were recaptured during this study: *Elliptio pullata* (n = 5), *Fusconaia burkei* (n = 1), *Hamiota australis* (n = 19), *Obovaria choctawensis* (n = 1), *Strophitus williamsi* (n = 1), *Villosa lienosa* (n = 60), and *Villosa vibex* (n = 3). Our sampling included individuals ranging from 24 mm to 80 mm in total length and two species (*F. burkei* and *H. australis*) protected by the U.S. Endangered Species Act. All data utilized in this study are publicly available We have provided access to our dataset on ScienceBase (https://doi.org/10.5066/P90VU8EN)[42].

Survivorship was assessed by how many individuals were recaptured alive after gill sample collection. We observed high survivorship (97%) during the study with some mortality, possibly contributable to predation, indicated by on-site observations. Results showed around 51% of

individuals (46 of 90) were found to stay gravid between consecutive sampling events. Another 10% of individuals (9 of 90) were found gravid, recaptured not gravid, and found gravid again. About 39% of individuals (35 of 90) in this study were found gravid, a gill sample was taken, but when recaptured again throughout the year, they were never found gravid a second time. The results indicate that the protocol described here is neither lethal nor sub-lethal and does not substantially disturb the current brooding period after the gill was sampled.

Although sample sizes in this study are unequal across species, results from this study highlight the beneficial and practical applications of this protocol. The gravidity calendar for *V. lienosa* illustrates gravid females brooding FDG were found in almost every month of the year except August, when only females brooding EGG were found (**Figure 5A**). Female *H. australis* were found not gravid (NG) in July, August, and December. A larger proportion of females were brooding FDG in January and February but were also found in October and November (**Figure 5B**). No individuals of *E. pullata* were found brooding FDG although females were brooding EGG from May to June, and one gravid female recorded (GFR) in June (**Figure 5C**). The only gravid *F. burkei* female was found GFR in June and recaptured NG in July. The same *O. choctawensis* individual was collected FDG in February and recaptured NG in July. Only one *S. williamsi* was found and was recaptured three times. This female was found FDG in March, NG in May, GFR in June, and EGG in August (**Figure 5C**). Gravid females of *V. vibex* brooding FDG were found between February and June (**Figure 5C**).

[**Figure 5** shown here]

FIGURE AND TABLE LEGENDS:

Figure 1: Gently prying individuals open. To check gravidity of a live mussel, gently pry open the valves with thumbs (**A**) or cautiously use a speculum or reverse pliers to pry open the valves (**B**). Review step 2.3 in the protocol for cautions associated with this method.

Figure 2: How to identify a gravid female. Female mussel marsupial gills appear inflated when the female is gravid and brooding. Photos A and C show gills from a lateral perspective while photos B and D provide a ventral view of the gills. Red boxes outline the gills to highlight the differences between a gravid (A/B) and not gravid (C/D) female *Lampsilis straminea* mussel. The total lengths of the individuals are 79 mm (A/B) and 88 mm (C/D).

Figure 3: Example of a field gravidity datasheet. Accurate data reporting is necessary if a gill sample is taken to produce reliable information. This is an example of a field datasheet with the minimum fields and extra abiotic parameters to be collected along with each gill sample. For more comprehensive information, please see step 4.1 in the protocol.

Figure 4: Representations for various stages of glochidia development in the marsupial gills. (**A**) Egg masses (EGG) have a membrane that makes eggs clump together. Within each egg membrane there is an opaque spherical mass of differentiating cells. The opaque spherical mass may split into multiple spherical masses during early cell division but should still be recorded as EGG until

a distinct bivalve shape is observed. (**B**) Immature glochidia (IMG) have a distinct bivalve-shaped mass contained within the egg membrane. (**C**) Developing glochidia (DG) have a distinct bivalve shape, no egg membrane, and unorganized tissue inside, often fuzzy in appearance. Developing glochidia (DG) are not reactive when exposed to NaCl and classified as 'DG(T)' when data are recorded. (**D**) Fully developed glochidia (FDG) have the distinct bivalve shape and obvious adductor muscle tissue allowing glochidia to close. Fully developed glochidia (FDG) are often observed as two open valves after preservation. Two open valves will usually snap closed, or snap open and closed, when exposed to NaCl and are classified as 'FDG(T)'.

Figure 5: Results of the study in Bruce Creek, FL displayed in a gravidity calendar format. (**A**) Gravidity calendar for *Villosa lienosa* captures/recaptures. (**B**) Gravidity calendar for *Hamiota australis* captures/recaptures. (**C**) Gravidity calendars for all species with less than 10 individuals sampled. The y-axis includes abbreviations for the months January (Ja), February (F), March (Mr), May (My), June (Jn), July (Jl) August (A), October (O), November (N) and December (D).

DISCUSSION:

Significance

Conservation of imperiled species depends on successful recruitment within extant populations. In some cases, artificial propagation may be necessary to augment recruitment of these at-risk populations. This requires researchers being informed on the timing of active reproduction for each species and possibly applying different methodologies or management practices to mitigate impact on recruitment. As an imperiled group of organisms, it is paramount to establish a standardized and non-lethal approach for studying reproductive habits, and to provide a platform on which to compile and visualize data to inform the scientific community with the most up-to-date information available. This study provides a step-by-step protocol to ensure precautions are taken, and gill contents can be adequately sampled and evaluated from female mussels. This protocol was tested for lethal and non-lethal effects, allowing researchers and managers to responsibly implement this methodology. We also developed a suite of database management tools and applications to facilitate compilation of gravidity information on a publicly available, user-friendly dashboard. Studies on epidemiology, glochidia morphology, life history, phylogenetics, propagation, and translocations can all benefit and utilize this repository of temporal gravidity information for all species of freshwater mussels.

This study alone supported previous studies' findings of some species reproductive habits but also revealed novel information on others. Although *V. vibex* was collected in fewer numbers than *V. lienosa*, similarities can be found between the two based on the gravidity data. Both species of *Villosa* seem to brood fully developed glochidia during a large portion of the year, which characterizes them as an overwintering brooder. This is consistent with previous studies on other *Villosa* species[43-45]. The results of this study suggest *H. australis* can be found gravid from October and overwintering into June, except no captures were found gravid in December. A previously published study identified congener *H. altilis* with a gravidity period of four months, March through June[46,47]. This finding illustrates a longer gravidity period than previously thought

and generally groups *H. australis* as an overwintering brooder. As federally protected species, varying brooding periods for *H. altilis* and *H. australis* could impact management decisions to better protect populations during reproductively active times. *Elliptio pullata* were only found gravid with EGG in May and June which corresponds to their characterization as a tachytictic species with a very short brooding period[24,48-50]. As data are compiled on *Elliptio* species using this protocol, detailed information can make field efforts more efficient when certain glochidial development stages are targeted, since glochidia are only found a few months out of the year. Inference from the other species with lower sample sizes is limited but as data are compiled into the database, higher sample sizes will give insight into reproductive habits of additional mussel species.

Procedural comments

Freshwater mussels and their glochidia are known to be susceptible to anthropogenic stressors[10,35]. During gravidity inspection, the mussel valves may not be easy to open, and carelessly forcing the valves open can cause unintentional harm and result in stress or mortality. Some fragile-shelled species (e.g., species of *Anodonta*, *Leptodea*, *Utterbackia*, etc.) and smaller sized individuals may have very fragile shells and weak adductor muscles that can break and tear easily. Gill sampling could be considered a stressor if handling is not done responsibly and with caution. A previous study found that handling and aerial exposure of mussels during reproductively active times may cause various physiological stress, including premature release of gill contents[34]. However, a study utilizing a similar methodology as described here, found handling gravid female mussels during gill sampling did not interrupt the present brood or cause premature release in both short- and long-term brooding species[16]. Furthermore, a sterile syringe needs to be used during this protocol to prevent any unintended infection or cross contamination when puncturing gills of multiple individuals. Additionally, glochidia are fragile and broods can be matured and stressed but not expelled. Mature glochidia in poor health can result in fewer individuals reacting to salt tests[35]. When making the distinction between DG(T) and FDG(T) it is important to salt test with a large sample size, make notes on observations to carefully identify distinctions between DG and FDG glochidia using the descriptions provided in this study. When proper care is taken, the minimal stress induced by this procedure can allow for female mussels to continue brooding glochidia naturally and reduce impacts on recruitment in the population.

Additional data can be recorded to supplement the database and provide broad context for reproductive habits of freshwater mussels. Some species (e.g., species of *Fusconaia*), have been observed to have gills of different colors based on the development stage of the glochidia[51]. During an initial gravidity check of the female, a description of the gill color may be included in the reported data to allow for future investigation. Also, at this point in the protocol, researchers can note whether the brooding female was found brooding glochidia in the two outer gills (ectobranchus), two inner gills (endobranchus), or all four gills (tetragenous)[17]. This information can be added to FMGA and help fill in data gaps regarding brooding for each species investigated. Environmental conditions, specifically water temperature, can be collected and recorded in the field for a more comprehensive observation of the gravidity status and timing of species at various latitudinal ranges. Research shows that environmental parameters, such as temperature,

photoperiod, flow rate, and food availability, may induce reproductive events in freshwater mussels[52-56]. Additional fields may be added to the database as they are submitted to promote future research on abiotic factors influencing gravidity. A capture-mark-recapture modification modeled after our study can also be added to this protocol, which would allow researchers to monitor a specific mussel's reproductive habits and reveal information on multiple broods per year.

The accuracy of information in the FMGA depends on the source. For example, misidentification of freshwater mussels is common due to many species having similar external characteristics that make it difficult to distinguish between species[57]. A gill sample from a misidentified individual could create confusion and false information for a species' brooding period. If a gill sample is taken, photographs should be taken of inside both valves (if individual is not alive), outside of right valve, and the umbo (hinge where two valves connect) and submitted with gravidity data through the desktop site or mobile application. We also welcome photos of the gill contents. Within the submission forms there is a drop-down menu enabling the collector to indicate their level of confidence regarding species identification. Before the record is validated, this information will be taken into consideration when checking collector identification against plausible distribution, etc. Due to the high degree of intraspecific morphological variation in species of Unionidae, submission of tissue samples is encouraged and may be necessary to facilitate molecular identification.

Future implications

As a non-lethal method, this protocol can be applied to both common and imperiled species. The gravidity calendars for imperiled species can assist conservation managers involved with endangered species legislation and recovery planning by providing information on time periods when species are reproductively active. The state and federal agencies that manage at-risk species can better advise permit allocations for times when the species is not vulnerable and reproducing, and even limit the harvest of host fish during times mussels are brooding fully developed glochidia. Additionally, field surveys can target species during non-reproductive periods to minimize impact to recruitment processes. The publicly accessible database, FMGA, provides a tool for researchers and managers to obtain important reproductive information on any target freshwater mussel species. The database will also highlight data gaps, encouraging further research on species-specific brooding patterns. Since understanding a species reproductive pattern allows for adequate management decisions to be implemented, we hope that our protocol and database facilitate future freshwater mussel research, conservation, and recovery.

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DISCLOSURES:

The authors have nothing to disclose.

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Fig1

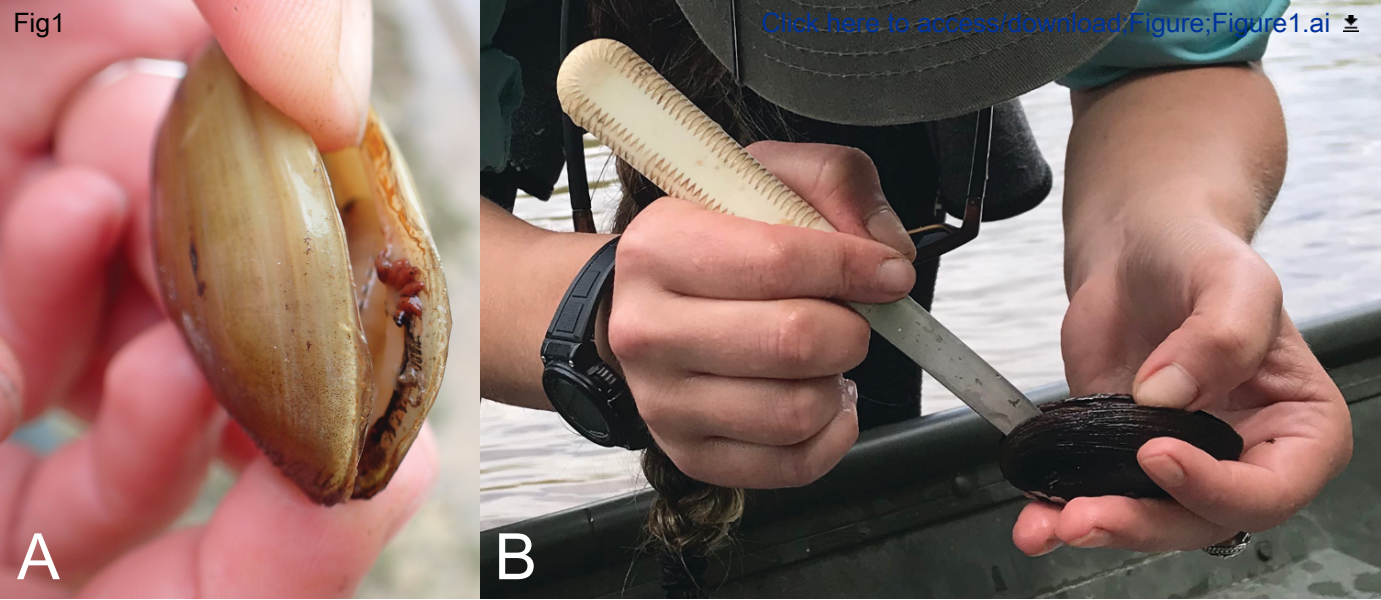

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Fig2

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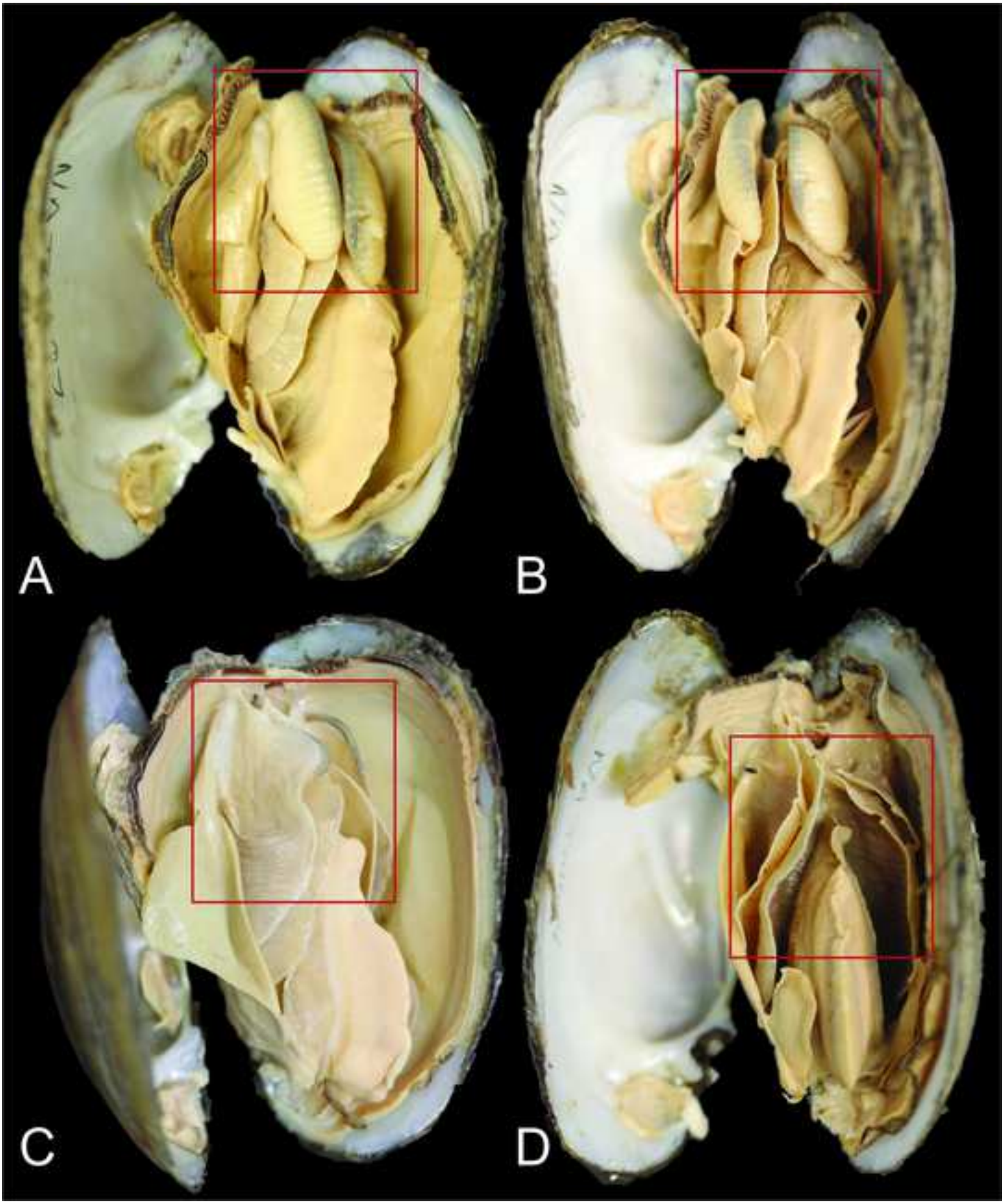

Fig3

USGS - WARC - EYC Mussel Gravidity Datasheet (Revised 07/12/2019) Page of

Field #: Lat: Long: Date:

State: County: Drainage: Waterbody:

Locality: Collector(s):

	Pass (1,2,or 3)	Collector intials	Genus	Species	Recap Y/N	Tag #	Length (mm)	Sex	Gravid	Tube ID	Gravidity Code	Notes
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Water Temperature Flow Rate Conductivity

Sex: male = **M,** female = **F**, undetermined = **U**; **Gravid**: yes = **Y** , no = **N**, partially gravid = **P**, not checked = **NC**
Gravidity Code (USGS only): eggs only (**EGG**), immature glochidia (**IMG**), developing glochidia (**DG**),
fully developed glochidia (**FDG**), glochidia reactive to NaCL (**FDG (T)**)

Other Comments:

Fig4

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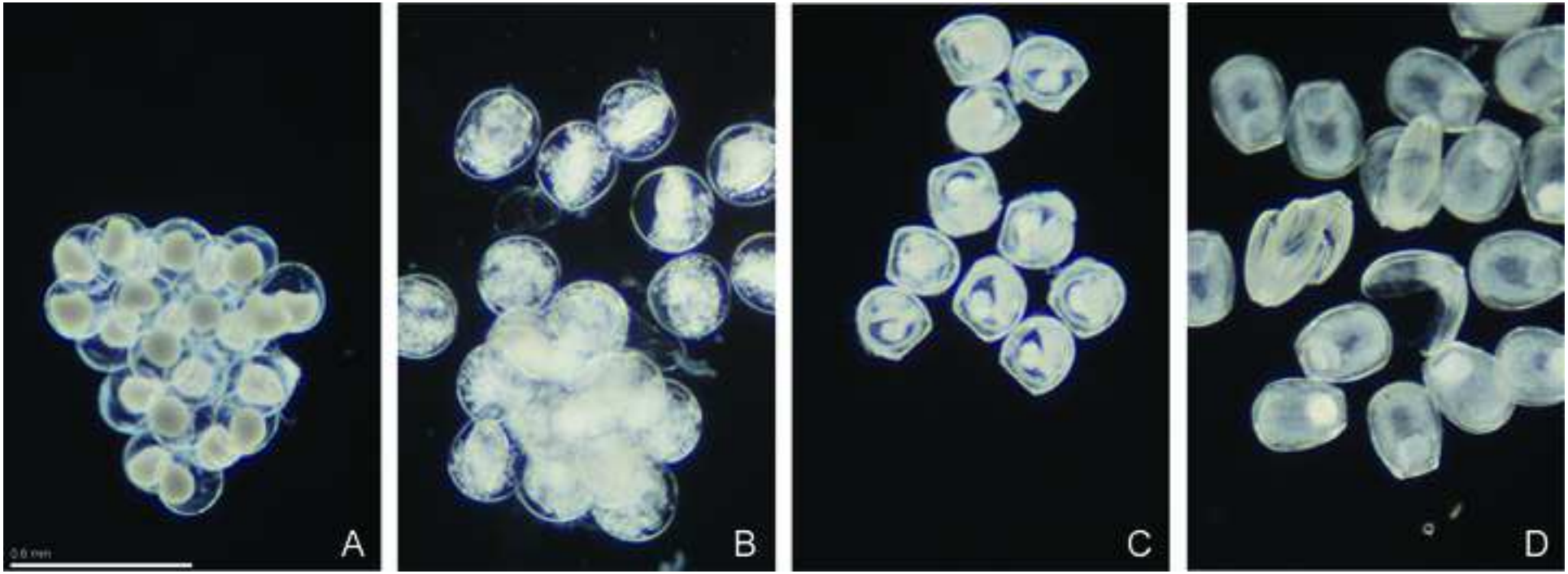

Fig5Gravidity Calendar for Villosa lienosa

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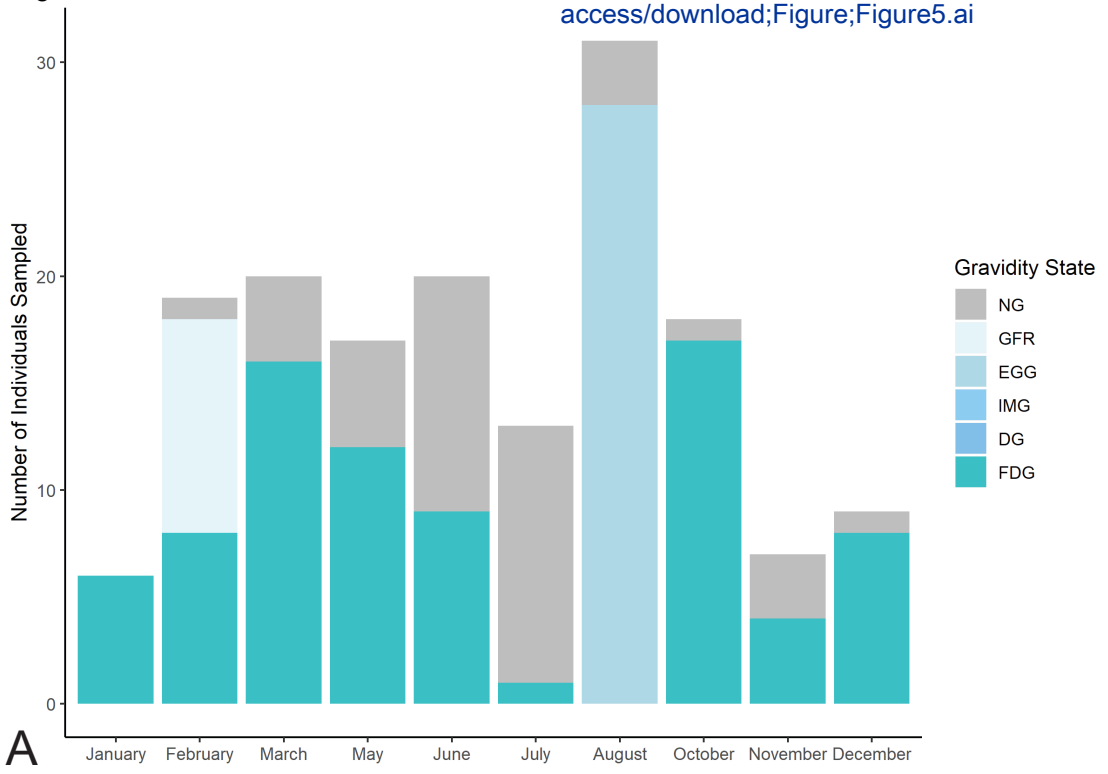
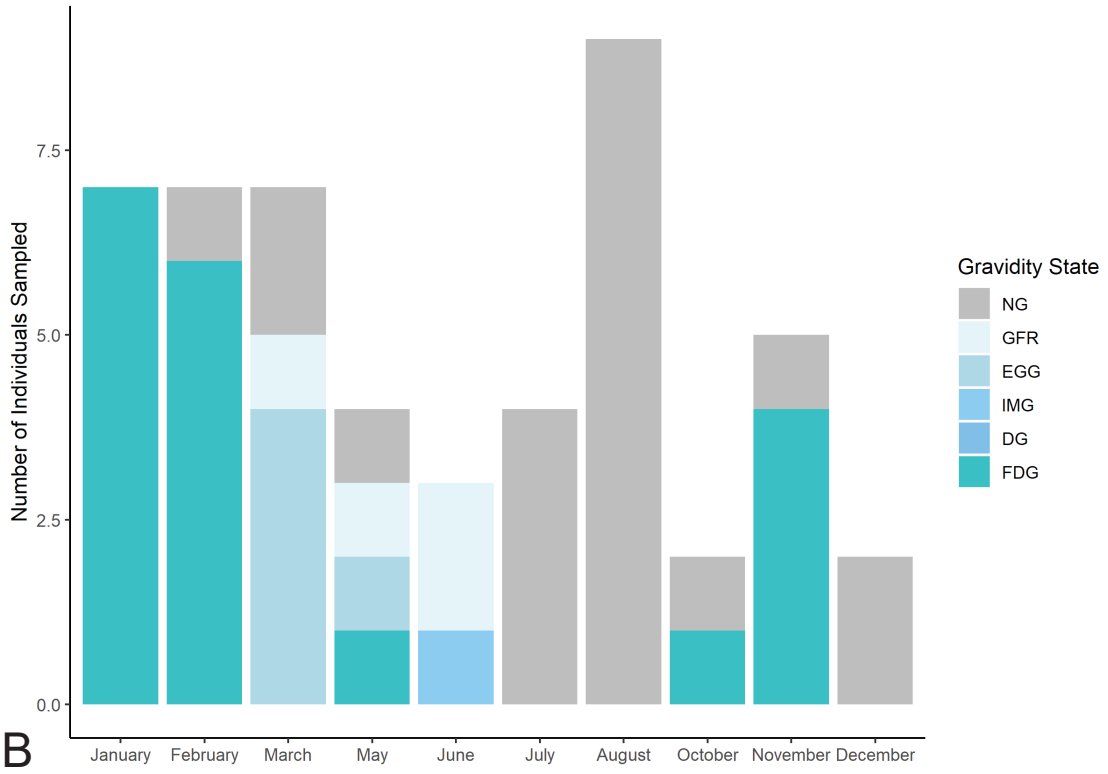
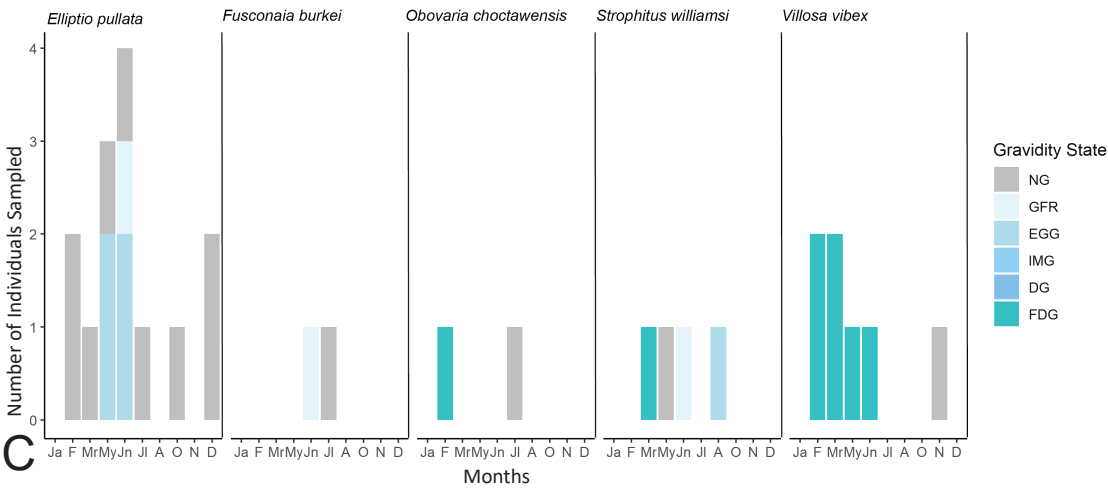


A

Gravidity Calendar for Hamiota australis



B



C

Name of Material/ Equipment	Company	Catalog Number
1.5 mL snap cap centrifuge tubes	USA Scientific	1615-5510
20 G needle on 10 mL disposable syringe	Exelint International	26255
Dissecting Microscope	any	any
Marking Pen	Fisher Scientific	13-379-4
Molecular grade ethanol	any	any
Paper	any	any
Pen/pencil	any	any
Petri dish	DWK Life Sciences (Kimble)	23000-9050
Sodium Chloride	any	any
Speculum	any	any
Sterile water	any	any
Super glue	Gorilla	Gorilla super glue gel
Tags	Hallprint	FPN 8x4
Transfer Pipet	Thermo Scientific Samco	225
Tweezers	any	any
Waterproof paper	RainWriter	any
Wooden pick	any	any

Comments/Description

Snap cap tubes are important in the field so the loose screw cap is not lost.

sterile 10 mL disposable syringe with needle Model: 10ml Luer Lock Tip W/20G X 1 1/2"

This is what we used but any marker that can write on small plastic tubes will do. This one is fairly ethanol and water proof.
Needed if preserving gill contents. Non-denatured 95% is needed for genetic work, 70% is needed for SEM imaging work.
Needed to record information on samples collected.
If in the field, better to write on waterproof paper with pencil so it doesn't smear. If in the museum/lab, any writing utensil is fine.
This is what we used but any petri dish available is fine. It is nicer to have the taller walls in case too much water is used.
Needed for NaCl test for reactive glochidia. Preserved samples do not need this.
Only needed if you want help opening the valves of a live mussel.
Added to gill samples to be evaluated for reactivity within 24 hours of collection.

Used to apply tags and only needed if conducting a capture-mark-recapture study.
Only needed if conducting a capture-mark-recapture study.
This is what we use but any transfer pipet or squirt bottle is applicable.
Needed to move crystals of NaCl for salt test. Preserved samples do not need this.
Only needed if conducting work in the field. This allows you to record information on each individual gill contents are extracted from.
Only needed if you want help opening the valves of a live mussel.

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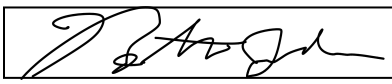
Signature:	
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Editorial Board - *JoVE*

Dear Editor,

On behalf of my co-authors, I am submitting a modified version of the manuscript entitled **Standardizing a non-lethal method for characterizing the reproductive status and larval development of freshwater mussels (Bivalvia: Unionoida)** [JoVE60224], for additional consideration by *JoVE*. Based on suggestions and feedback from the reviewers, we made additions to the manuscript and revised according to the editorial comments. In addition to the journal's peer review and editorial comments, this manuscript has gone through an internal review and has been approved for dissemination by the bureau officers of the U.S. Geological Survey. Our responses to editorial and reviewer feedback are detailed at the end of this letter in bold text.

In our investigation, we describe a standardized technique for evaluating the reproductive status and larval development of freshwater mussels. We carefully evaluated lethal and sub-lethal impacts of our protocol on a suite of freshwater mussel species using a mark-recapture study and found no impacts to survivorship and reproduction. To accompany our standardized sampling methodology, we developed a publicly accessible database to serve as the repository for information on timing of reproductive habits and larval development to facilitate future freshwater mussel research, conservation, and recovery.

Our technique can be applied to approximately 821 species of freshwater bivalves belonging to three families (Unionidae, Margaritiferidae, and Hyriidae), which are distributed on every continent except Antarctica and have diversity hotspots in North America and southeastern Asia. Freshwater mussels are of high conservation importance globally and our study provides valuable resources to help researchers and managers with conservation assessments, designation and protection of critical habitats, and development of effective conservation and recovery strategies.

Requests from our partners and collaborators to develop a video illustrating our protocol is what originally led us to explore JoVE as a publication option. Subsequent research on the journal, the option for open access, broad readership, and swift review and publication timelines were additional reasons we selected *JoVE* as the outlet for our work. We thank you for your time and consideration and understand costs associated with publication in *JoVE* and will pay all charges prior to publication. Additionally, the US Government requires that all data and supplemental tables will be made publicly available using ScienceBase (http://www.sciencebase.gov) once our article has been reviewed. We welcome *JoVE* to host the supplemental files as well and are amenable to the journal's preferences. Please contact either myself or the lead author with any correspondence relating to this manuscript using our contact information provided below.

Nathan Johnson, Ph (352) 264-3574, email najohnson@usgs.gov
Caitlin Beaver, Ph (352) 264-3573, email cbeaver@usgs.gov

Sincerely,

Nathan Johnson

Editorial Comments:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

This has been done.

- Avoid the use of pronouns "you", "your".

All instances have been removed.

- Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

The protocol is highlighted accordingly.

- Please confirm that the animals will be available for filming.

We confirm that animals will be available for filming by the date the editors give for production.

- 2) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
- 3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
- 4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
- 5) Notes cannot be filmed and should be excluded from highlighting.

The protocol is highlighted accordingly.

- Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We believe the discussion addresses these key points and is no longer than the maximum number of paragraphs.

- Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are ArcGIS®,
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All commercial sounding language has been removed from the manuscript.

- If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All of our figures and tables are original and have not been previously published.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

- 28-29 - too general statement, pls. substitute with more specific information about monitoring of reproduction

We edited the sentence accordingly.

Major Concerns:

I carefully read the MS with the title "Standardizing a non-lethal method for characterizing the reproductive status and larval development of freshwater mussels (Bivalvia: Unionoida)". The MS deals with a topic potentially attractive for broader readers audience and well corresponds with the focus of the JoVe journal. The MS is well structured and is clearly formulated what authors want to be presented. The protocol represents a useful tool with a potential to unify monitoring of mussels for the purpose of species protection. The methods are properly described and repeatable. The results are convincing. However, I found some sections of the text that need improvements. I recommend to accept the MS for publication after proposed revisions of the text; below are formulated some comments.

- From the methodological point of view I do not recommend to use a knife. Even if it is dull, it can cause the injury of an individual. In addition, its use does not determine the width to which shell should be opened, which is specie specific. I would recommend to use calibrated key with the scale, where jaws of the key precisely open the mussels shell to safe level of the tested specie.

Our field crews use dulled clam knifes or reverse pliers, so we reworded the methods to be more specific. We agree with the reviewer's concerns and have added more qualifiers, however, sometimes a tool is required to open a mussel. We are not aware of any existing scale or species-specific ratio of length/age/size to valve opening. We believe with variable intraspecies size and shapes that a standardized scale may not be applicable. This would be an interesting research question but is beyond the scope of this study.

Minor Concerns:

Introduction

- 34-35 - similarly, too general, even naïve

We edited the sentence accordingly.

- 59-73 - well structured paragraph, however, missing is information that decreasing abundance of host fish species represents an important threat for mussels reproduction

Thank you. This information was added.

- 92-108 - Why do you present only American references? It is a little bit disqualifying for the MS. I understand that the protocol is designed for the Federal Agency of nature protection and museums in the USA, however, in other parts of the MS you balanced the use of international literature sources, and you referred to international studies…

Thank you for catching this. We now include citations of international literature in this paragraph.

- 116-134 - yes, description of the goals is important, but the paragraph needs to be simplified and shortened

We edited the paragraph accordingly.

Reviewer #2:

Manuscript Summary:

In this paper, the authors describe and standardize a non-lethal technique for characterizing reproductive status and larval development of freshwater mussels. The manuscript makes a valuable contribution to freshwater mussel ecology and conservation. The description of the technique is great, pictures (for the most part) are excellent, and the description/inclusion of a reporting database is invaluable. I have used this technique in the past and have found it extremely useful. The description/refinement/standardization presented in this paper will greatly increase the value of the methodology and hopefully allow it to become more widespread. This has the potential to become a highly valuable and widely cited reference. Concerns are as follows:

Major Concerns:

- Need to cite/refer to previous studies using this technique - results of previous studies will strengthen conclusions of this paper

We referred to previous studies that used this technique in the introduction, protocol and discussion sections of this paper.

- Need to temper conclusions regarding the lack of effects of gill extraction on brooding females

Conclusions and results were tempered to reduce the confusion but citations were put in place to further explain the validation of this technique.

Minor Concerns:

Introduction

- Line 98. Suggest rewording as "..researchers to generally describe brooding females as 'gravid' which does not fully encompass…"

Agreed. Thank you!

- General: suggest that the authors cite and give a brief description of the origin of the technique they are describing/formalizing/testing and clarify that it is not a 'new' technique. My lab has used this technique in the past and this is the way that I understand its basic origin:
    - Saha and Layzer (2008) described a non-lethal technique using a syringe to extract gonadal fluid to identify sex and gamete stage. This technique was further evaluated and validated by Tsakiris et al. (2016).
    - 2) Gonad technique was adapted for use in gills by Gascho Landis et al. (2016). Multi-stage disruption of freshwater mussel reproduction by high suspended solids in short- and long-term brooders. Freshwater Biology 61(2):1-10: Described a non-lethal technique using a syringe to extract eggs/glochidia from mussel gills (adapted from Saha and Layzer 2008). They defined four categories (empty gills, unfertilized eggs, embryos, glochidia), verified that the technique was non-lethal and did not prevent mussels from becoming retaining embryos or becoming gravid again after sampling (i.e. table 2), and used data to define the spawning period of L. subrostrata (i.e. Fig. 2). This is very similar to some of the main goals of the current study. To strengthen the current manuscript I'd suggest they describe how they have refined/improved the original methodology, and use the results described in Gascho Landis et al. (2016) to further support the conclusions of the current study- which are largely based on small sample sizes for the majority of species, and larger sample sizes for a single species. Conclusions regarding the value and effectiveness of this technique are very similar between the two studies, so I think that this will help strengthen the current paper and help alleviate concerns arising from small sample sizes.

The clarification has been made in the introduction section and the Gascho Landis et al (2016) study was cited in the discussion to support findings.

Protocol

- Line 156-158. Suggest listing general taxonomic group(s) that are ectbranchus vs tetragenous so readers will know which group their study species is likely to fall into.

Information regarding brooding for all taxa is unavailable and beyond the scope of this study. There are approximately 300 species just in US and Canada and given this protocol is applicable to all unionids and hyriids, we are unable to determine which taxa are ectobranchus vs endobranchus vs tetragenous. We agree this would be great information to publish. In our research, we have noticed high levels of both intraspecific and intrageneric variation with respect to brooding. We don't feel that brooding is conserved even at the tribal level (e.g. species in Quadrulini can be ectobanchus while others brood in all four gills). Given the levels of variation and incomplete knowledge regarding brooding, we have modified our protocol to encourage researchers to make note of whether an animal is ectobranchus, endobranchus, or tetragenous. This information, when provided, will be added into a field for the online database.

- Line 160 (Fig 1) - suggest better picture of live mussel showing swollen gills. Swollen gills are not evident in Fig 1a. It is important that readers can see an example of what swollen gills look like in live mussels that are only partially open. Even better would be to show an example of both an ectobranchus and a tetragenous gravid female

Figure 1a was an illustration of how to open valves of a live mussel. Figure 2a was used as an illustration for what gravid gills may look like. Gravid individuals will also be included in our JoVE video.

- Line 209 -210. So..water tube contents are not "sucked out" with a syringe, but are scooped out using the syringe tip like a trowel..is that correct? Does this seem to be less intrusive? I have always used a syringe to suck out gill fluid, but maybe it is better to do it this way.

That is correct. Our lab has tried out many different techniques to remove glochidia from live mussels. It does seem less intrusive to scoop. When "sucking out" water tube contents, our lab has found that it becomes difficult to get the glochidia out of the syringe, involving more water be flushed through the syringe to expel all of the sample and sometimes damaging the glochidia, which could skew viability testing and overall condition of the sample observation. Although we did not specifically test the different methods of gill sampling, based on our experience, we believe scooping to be the less intrusive and most preferred method.

- Line 251 "..Sodium Chloride (NaCl) test.." Authors should cite Fritts et al. 2014. Assessment of toxicity test endpoints for freshwater mussel larvae (glochidia). Environmental Toxicology 33(1):199-207 for a description/evaluation of the salt test technique. This is especially important since glochidia that do not snap shut may be in poor health, and not necessarily "developing".

The in-text citation was added, thank you!

- Lines 255-256. Suggest revising as "...glochidia of some species...NaCl and the salt test is not applicable to these species." This rewording depends on the goals of the authors with regards to the salt test. If it is to simply see if ANY glochidia close when exposed to NaCl, then the original wording may be ok, but if the goal is to actually calculate percent viability using the salt test, then the salt test is not applicable to "snappers". I have previously run into this issue with snapping glochidia.

We reworded the step in the protocol to clarify the purpose of the salt test. We think getting too involved in the intricacies of percent viability may confuse the readers. We are trying to keep it as simple as possible for the novices who may be utilizing this protocol for the first time.

- Line 268 Does this refer simply to presence/absence, or do the authors want an estimate of proportions within each stage?

Simply presence/absence. Clarification was made in the protocol.

- Line 271-273. The proportion of glochidia that react to NaCl can vary widely among broods. Similar to the previous comment, are the authors simply asking if ANY glochidia snap shut or are they asking for proportions or numbers in each category?

We are asking if ANY glochidia snap shut. The protocol was modified with this clarification.

- Line310 - 313. Results are primarily based on V. lienosa recaptures. This species is a long term brooder and relatively insensitive to handling. Short term brooders are much more sensitive to handling and more likely to abort broods after gill extraction. Based on this strong bias towards long-term brooders, should the authors apply their conclusions to all mussel taxa, or primarily to short term brooders. As mentioned previously, authors could strengthen conclusions by referring to previous studies (i.e. Gascho Landis et al. 2016) who used a very similar technique on a short-term brooder (R. ebenus) that is sensitive to handling.

We added in support statements and cited Gascho Landis et al. 2016 in the discussion.

- Line 317. How was survivorship and mortality assessed?

Clarification was made.

- Lines 318-322 - Authors lump all taxa together for these conclusions, but as mentioned previously, the vast majority of results seem to be coming from a single, long-term brooding species. Authors should specifically mention results from short-term (handling sensitive) species that support their conclusions.

We feel with the small sample sizes that this will not give much information. We have revised our results and adding to the discussion related citations that support our findings.

- Line 320 "...unlikely to induce premature release..." This conclusion is based on 46 of 90 remaining gravid between subsequent sampling events. Isn't it just as valid to say that results suggest the protocol is likely to induce premature release of gill contents about half the time, since roughly half of the mussels re-examined were no longer gravid?

We agree and have revised the results and addressed these concerns in the discussion.

- Line 322 "...did not inhibit females from becoming gravid.." Similar concern. Only 10% that were no longer gravid became gravid again. Couldn't this suggest that there was a strong inhibition? For this and previous comments, authors should either temper their conclusions clarify how they came to these conclusions.

We agree and have revised the results and addressed these concerns in the discussion.

- Lines 323 - 324: "..about 38.9%...were found gravid...were never found gravid a second time." Doesn't this contradict the next sentence stating that the protocol does not disturb the current brooding period? Overall, I totally agree with the authors that this is a valuable, non-lethal technique but I don't think they clearly demonstrate that it does not disturb brooding. I think their results, similar to the Gascho Landis study, show that the technique might disturb brooding somewhat, but a "substantial" (clarify based on results) proportion of individuals are likely to retain broods and/or become gravid again after sampling. Effects are likely to be relatively minor...definitely not catastrophic.

We agree and have revised the results and addressed these concerns in the discussion.

- Lines 328 - 330: Not sure I understand how these two sentences go together.

We edited the sentence accordingly.

- Line 372 "Developing glochidia..." Authors should clarify that mature glochidia in poor health may also not be reactive. A lack of reaction to salt would not distinguish between "developing glochidia" and mature glochidia in poor health. This is an important point to make. According to the protocol as described here, a female with a brood that has been subjected to severe stress (or is simply 'old') would be scored as 'developing' when that might not be the case at all.

These concerns have been addressed in the discussion. The figure legend text was revised as well.