Evaluation of a Fluorescent Dye Assay to Assess Glochidial Health

by

Kathryn Marie Mitchell

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements of the Degree of
Master of Science

Auburn, Alabama
May 8, 2016

Keywords: Freshwater mussel, fluorescein diacetate, propidium idodide, cryopreservation

Approved by

James A. Stoeckel, Chair, Associate Professor of Fisheries, Aquaculture and Aquatic Sciences
William H. Daniels, Associate Professor of Fisheries, Aquaculture and Aquatic Sciences
Jeffery Turhune, Associate Professor of Fisheries, Aquaculture and Aquatic Sciences

Abstract

Unionid mussels have the greatest diversity in North America but have suffered high extinction and imperilment rates due to habitat loss, fragmentation, and deterioration. Unionid mussels have a parasitic larval stage (glochidium) that requires a fish host to metamorphose into a juvenile. The current technique of evaluating glochidia viability is known as the salt test, which predicts metamorphosis success based on the shell closure in response to salt (NaCl). However it is only useful on open glochidia. This study evaluated a dual staining assay that involves fluorescein diacetate (FDA) and propidium idodide (PI); FDA can be combined with PI to evaluate membrane integrity and physiological state of open and closed glochidia. Proportion live, questionable, and dead glochidia can be evaluated using fluorescent microscopy; living glochidia will fluoresce green and dead glochidia will fluoresce red. Questionable glochidia (live but heavily damaged) exhibited green and red fluorescence. The dye assay was comparable to that of the salt test when predicting metamorphosis success. It appears to be a useful tool for ecotoxicology testing and could be a key technique in development of successful cryopreservation techniques for glochidia. Cryopreservation could preserve genetic diversity and reduce genetic bottles in future generations of threatened and endangered freshwater mussels.

Acknowledgments

I would like to first thank my advisor James A. Stoeckel for giving me the opportunity to be a graduate student under his guidance and also for allowing me to participate in a wide array of experiments and field expeditions during my time as a graduate student. Many people provided assistance to me while working on my thesis and I would like to sincerely thank all of them. I would like to thank my committee members, Dr. William Daniels, and Dr. Jeffery Terhune for their contributions to the analysis and writing of this thesis as well as assisting in my graduate school admission. I would like to thank William Wayman and Jaci Zelko from USFWS Warm Springs Fish Technology Center for their help with cryopreservation and *in vitro* transformation. I would like to thank Karen Veverica for supplying the bluegills used in this study and for providing funding and hands-on aquaculture experience during my time as a graduate student. I would like to thank Lindsay White for her assistance with identifying bluegills and editing of this thesis. I would like to thank Ben Station for his assistance with statistics. Finally I would like to thank my parents; Brent and Melanie for their unconditional love, support, and encouragement during my time as a graduate student.

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Chapter 1 Evaluation of a new technique to evaluate glochidial health

Introduction:

Freshwater mussels belong to the order Unionoida, which includes more than 850 species from 6 families. Unionid mussels have worldwide distribution with the greatest diversity (306 species) occurring in North America (Bogan 2008). Unionids have an unusual life cycle that includes a parasitic larval stage (glochidia), which requires a vertebrate host, usually a fish (Barnhart et al. 2008). Some unionids are specialists and can transform on only a small number of fish species while others are generalist, using a wide range of fish species. In the laboratory, glochidia can also be transformed *in vitro*, which is an artificial propagation method that does not require a host for mussel larvae to metamorphose into juveniles. Instead the larval cycle is completed in a Petri dish containing cell culture media, serum, and antibiotics (Lima et al. 2012). *In vitro* transformation has been successful in 42 species of unionids, several which are federally endangered (Hudson and Shelbourne 1990; Isom and Hudson 1982; Keller and Zam 1990; Owen et al. 2010; Uthaiwan et al. 2001; Uthaiwan et al. 2002). Within the United States and Canada, over 200 unionid species are listed as vulnerable, imperiled, critically imperiled, possibly extinct, or presumed extinct by the Natural Heritage Network (Master et al. 2000). A current approach to conservation and restoration involves the production of juvenile mussels in hatcheries using live fish hosts. An alternative method currently being investigated by other researchers involves

long-term cryopreservation of mussel glochidia for later *in vitro* production of juveniles (Lima et al. 2012). Both approaches require an accurate assessment of glochidial health, as only healthy glochidia should be used to infest fish hosts, or frozen for long-term storage.

Early investigations into glochidial health by Lefevre and Curtis (1921) discovered exposure to various salt solutions or blood caused glochidia to close and Coker et al. (1921) suggested that the closing response was related to glochidia viability. Shell closure in response to salt (NaCl) is referred to as "the salt test" and is currently the primary method in which glochidial viability is assessed. Viability, as indicated by the salt test, is a good predictor of infectivity – the ability to attach to a host fish and metamorphose to the juvenile stage- when viability >90% (Fritts et al. 2014). Drawbacks of the salt test are that 1) the ability to predict infectivity decreases when viability is <90% and 2) it is applicable only to glochidia that are initially open. This is particularly problematic for studies involving cryopreservation in which otherwise healthy glochidia may close in response to chemicals and freezing, eliminating the usefulness of the salt test.

A previous study using fluorescent dyes was successful in assessing gamete quality of eastern oyster gametes that had been cryopreserved (Paniagua-Chavez et al. 2006). Dual staining utilizes a combination of dyes, one of which can penetrate intact cell membranes and another which can only penetrate damaged cell membranes. Fluorescein diacetate (FDA) is a

non-toxic, non-polar, non-fluorescent dye that has been used to assess quality of oyster eggs, plant cells, and other types of cells. FDA can penetrate intact membrane and be hydrolyzed by intercellular esterases to produce fluorescein, a polar fluorescent compound that collects in live, intact cells (Paniagua-Chavez et al 2006; Saruyama et al. 2013). Propidium idodide (PI) can be used to identify non-viable cells because it only penetrates damaged nuclear membranes and stain nucleic acids by inserting itself between base pairs. FDA emits green fluorescence with positive staining of viable cells and PI emits red fluorescence with positive staining of non-viable cells. FDA can be combined with PI to evaluate membrane integrity and physiological state. Viability can be evaluated using fluorescent microscopy; viable (living) cells will fluoresce green and non-viable (dead) cells will fluoresce red (Tsai et al. 2010).

The objectives of this study were as follows:

1) Determine whether the dye assay can distinguish between live and dead glochidia that are closed, as well as open.
2) Compare the relationships of the proportion of live glochidia (dye assay) and the proportion of viable glochidia (salt test) to increasing concentration of a reference toxicant.
3) Determine whether the dye assay is a better predictor of infectivity than the salt test.

Methods:

Study animals

Ligumia subrostrata, commonly known as the Pondmussel, grows to a length of 81 mm and is relatively thin and inflated. Age of sexual maturity is approximately 7 months. On males, the posterior margin is bluntly pointed and on females, it is obliquely truncate; females also become more concave with age. *L. subrostrata* usually occurs in areas with little to no current and in mud or sand substrate. It has been able to expand its range by becoming abundant in artificial ponds (Ortmann 1916). *Micropterus salmoides* (largemouth bass), *Lepomis cyanellus* (green sunfish), and *Lepomis humillus* (orangespotted sunfish) were all found to be successful laboratory hosts for *L. subrostrata* (Lefevre and Curtis 1912). *Lepomis gulosus* (warmouth) and *Lepomis macrochirus* (bluegill) were found to be hosts in natural infestations (Stern and Felder 1978). *L. subrostrata* are long-term brooders gravid from late summer or fall to the following spring and uses a mantle lure resembling a small invertebrate to attract fish hosts (Corey et al. 2006; Lefevre and Curtis 1912).

Gravid *L. subrostrata* were collected from earthen ponds at the South Auburn Fisheries Research Station (SAFRS) of Auburn University in March 2014, 2015 while water temperatures remained below 20°C. Mussels were held indoors in a 32’ X 18’ X 18” (170 liters) tank filled with artificial freshwater (AFW: 50 mg $CaCO_3$, 25 mg $CaCl_2$, 50 mg $NaHCO_3$ and 5 mL 30%

saltwater /L deionized water). Water was chilled to 10°C so that brooding females would retain their glochidia for several months beyond the end of the normal brooding season (Gascho Landis et al. 2012). Mussels were fed approximately 7 ml of Shellfish Diet 1800 (Reed Mariculture) 2-3 times a week and water quality was monitored weekly for TAN (total ammonia nitrogen), nitrite, and nitrate using Tetra® EasyStrips.

Lepomis macrochirus (bluegill) were caught in earthen ponds located at the North Auburn Research Station on April 9, 2015. These ponds did not contain mussels, thus the bluegill served as naïve hosts (O'Connell and Neves 1999). Fish were released into two outdoor, rectangular (3.5m L x 1.8m W x 0.9 H; 5,700 L) fiberglass tanks filled with water from a nearby earthen pond that was mussel free. Fish were fed commercial fish diet 2-3 times per week. Water quality was monitored weekly for TAN, nitrite, and nitrate using Tetra® EasyStrips.

Objective 1: *Ability of dye assay to differentiate live from dead glochidia.*

A stock solution of FDA was prepared by dissolving 5 mg of fluorescein diacetate into 1 mL acetone. Then prior to each assay, a working solution of FDA was prepared by adding 0.04 ml stock solution to 10 ml AFW (Tsai et al. 2010). Stock solutions were not utilized for PI, rather, a working solution was prepared prior to each assay by dissolving 4 mg in 50 mL of

phosphate buffered saline (PBS). The stock solution of FDA and working solution of PI will last up to 6 months if kept in the dark at 4°C (Tsai et al. 2010).

Glochidia were obtained by inserting a 22-guage needle into the marsupial gills of a gravid female and flushing glochidia with AFW into a beaker (Dodd et al. 2005). Glochidia viability was assessed using the salt test (Fritts et al. 2014). Only broods with >90% viability were used for dye assays, which means that >90% were open prior to staining.

Glochidia were flushed from one gravid female into a beaker containing AFW, mixed with plunger then pipetted onto ten depression slides (~20 glochidia/slide). One half of the slides were held on the counter at room temperature (~20°C) while the other half were frozen for 20 minutes and then thawed. All slides were then stained by adding 50 µL of both dyes to each slide. Glochidia were held in the dark for a minimum of 20 minutes to allow dyes to fully penetrate the cells and then examined under fluorescent microscopy. Phase microscopy (Ph3) was used to view both dyes; glochidia that fluoresced bright green under fluorescence 3 (FITC) but had little to no red fluorescence under fluorescence 4 (TRITC) were scored "live". Glochidia that fluoresced both green and red were scored "questionable" and glochidia that fluoresced red but had little to no green fluorescence were scored "dead" (Fig. 1).

To determine whether the dye assay could differentiate between live and dead glochidia that were closed prior to staining, the same procedure was followed except that a drop of salt

water (35 ppt) was added to each depression slide in order to cause all live, open glochidia to close. One half of the slides were held at room temperature (live closed) and the other half of the slides frozen at -4°C for 30 minutes and then thawed (dead closed). All slides were then stained, examined, and scored in the same way as the previous experiment.

Objective 2: *Response to a reference toxicant*

Glochidia were extracted from three gravid *L. subrostrata* using the previously described methodology, combined, and diluted to 2000 ml with AFW. Initial viability (0 hours) was estimated by applying the salt test to approximately 100 glochidia drawn from the mixed brood stock while initial proportion "live" evaluated using the fluorescent dye assay as previously described (4 slides; ~10 glochidia per slide) (Fritts et al. 2014). Glochidia subjected to the salt test or dye assay were subsequently discarded and not used further. The glochidial stock solution was then mixed with a plunger stirrer to avoid a vortex, and 100 ml of mixed solution added to each of the twenty (150 ml) beakers. Beakers were then randomly assigned to five treatments (four replicate beakers per treatment) and the appropriate amount of NaCl added to each beaker to bring it to the desired concentration (0.0, 0.25, 0.75, 2.25, 6.75 g NaCl / L).

At 4 and 24 hours, the contents of each beaker were evenly mixed with a plunger and subsamples withdrawn to evaluate viability (salt test) and proportion "live" (dye assay) of

exposed glochidia. The salt test was performed by pipetting a subsample of 50-100 glochidia from each beaker onto a counting tray, and then a dissecting microscope was used to count the number of open and closed glochidia. A few drops of 35 ppt artificial saltwater were then added to the sample and the number of open glochidia was counted again. The dye assay was performed by pipetting approximately ten glochidia from each beaker and onto a depression slide (one slide per beaker). To keep staining consistent among slides, excess water was pipetted from the depression before adding dyes. After slides had been held in the dark for a minimum of 20 minutes, glochidia were examined under a fluorescent microscope and scored as live, questionable, or dead. Proportion viable and proportion live were then regressed against NaCl concentrations (exponential decay, SigmaPlot 12.0).

Objective 3: *Ability to predict infectivity*

The ability to predict infectivity (ability of glochidia to attach to a host fish and metamorphose to the juvenile stage) was compared between the salt test and dye assay. On May 7, 2015, glochidia were extracted from five gravid *L. subrostrata* and pooled in a 2,000 mL beaker filled with AFW. The beaker of glochidia was held in a water bath at 15°C without aeration for 12 days and the salt test performed on subsamples of glochidia every 1-2 days using the previously described methodology. On Day 0 and each subsequent sampling day when

viability (salt test) had declined by ≥10%, the dye assay was conducted on additional subsamples to estimate percent live glochidia. Fish were inoculated using additional glochidia from the beaker.

To inoculate fish, an appropriate subsample of glochidia was taken from the beaker to make a 20-L inoculation bath at 4,000 glochidia/L (Fritts et al. 2014). The bath was aerated to keep glochidia in suspension, and five bluegills were held concurrently in the bath for 15 minutes to inoculate them with glochidia. Fish were then removed from the inoculation bath and held in a 20-L, aerated, AFW bath for an additional 15 minutes to rinse any free or poorly attached glochidia.

Fish were then placed in individual 1.5-L Aquatic Habitat (AHAB) tanks in a recirculating system containing AFW. AHAB systems are currently used by many mussel propagation facilities to raise glochidial mussels on host fish and subsequently collect young of year (YOY) mussels as they drop from fish following metamorphosis from the glochidial to juvenile stage. These systems are designed to be "self-cleaning" tanks, which work to passively flush sloughs and juveniles from the tank to an outside collection filter. Each AHAB tank had a 150-µm collection filter at its outflow that was rinsed and examined under a dissecting scope 3 times per week. The number of sloughed glochidia and juveniles was recorded for each individual fish on each collection day. Juveniles were differentiated from sloughed glochidia by

the presence of a foot and/or valve movement. Fish were monitored until no juveniles or sloughed glochidia were observed for three consecutive sampling days. Metamorphosis success was calculated by dividing the total number of juveniles by the total number of juveniles plus sloughed glochidia collected from each fish across all collection days.

The trial was then repeated starting on June 21st using the same methodology, in order to obtain infectivity values for seven additional % viability and % live estimates (Table 1). Metamorphosis success was then regressed against % viable (salt test) and % live (dye assay) using quadratic regressions (SigmaPlot 13.0).

Results:

Water quality was optimal in the AHABs and outdoor holding tanks throughout all experiments with total ammonia (NH_3-N) <1 mg/L, nitrites <1 mg/, and nitrate ≤10 mg/L.

Objective 1: *Ability of Dye Assay to differentiate live from dead glochidia*

The dye assay successfully differentiated between live and dead glochidia that were open prior to staining with 95 ± 5.7 percent of unfrozen glochidia scored as "live", 5 ± 5.7 percent scored as questionable, and none scored as dead. Conversely 100% of frozen then thawed glochidia were scored as dead (Fig. 2 top panel).

The dye assay also successfully differentiated between live and dead glochidia that were closed prior to staining. The assay yielded estimates of 92.5 ± 5, 7.5 ± 5, 0 percent live, questionable, and dead glochidia respectively for unfrozen glochidia and 100% dead (no live or questionable) for glochidia that were frozen and thawed prior to staining (Fig. 2 bottom panel).

Objective 2: *Effects of glochidial exposure to reference toxicant*

Percent viability, as measured by the salt test, exhibited a strong, significant pattern of exponential decay with increasing NaCl concentration over 4 hours [$R^2 = 0.96$, $P < 0.0001$, Y = 75.2143*exp(-2.2766*X)] and 24 hours [$R^2 = 0.98$, $P < 0.0001$, Y = 77.1779*exp(-3.9091*X)] periods. No glochidia were scored as viable at NaCl concentrations ≥ 2.25 g/L for either the 4-hour or 24-hour exposures. Furthermore, for the 24-hr exposure, only 10% of glochidia were viable at 0.75 g NaCl/L (Fig 3).

Percent live, as measured by the dye assay, also exhibited a strong, significant pattern of exponential decay with increasing concentration of the reference toxicant over 4 hour [$R^2 = 0.93$, $P < 0.0001$, Y = 97.8457*exp(-0.3696*X)] and 24 hour [$R^2 = 0.91$, $P < 0.0001$, Y = 96.6951*exp(-0.4553*X)] periods. In contrast to viability, glochidia were scored as >10% live at NaCl concentrations as high as 2.25 g/L for both the 4-hour and 24-hour exposures (Fig 3).

Objective 3: *Ability to predict infectivity*

Metamorphosis success declined in a significant, nonlinear fashion as the proportion of glochidia scored as viable (salt test) and live (dye assay) declined. Both assays exhibited nearly identical abilities to predict metamorphosis success based on R^2 values associated with quadratic regression (salt test $Y = 0.0294+0.1512*X+0.7697*X^2$, $R^2 = 0.818$, $P < 0.0001$; dye assay $Y = -0.3714+0.711*X+0.5767*X^2$, $R^2 = 0.819$, $P < 0.0001$). However, metamorphosis declined more steeply with declines in proportion live (dye assay) compared to declines in proportion viable (salt test). Metamorphosis success of glochidia used to inoculate fish declined to zero (no metamorphosis) when the proportion scored as live remained >0.4, but proportion of glochidia scored as viable was < 0.2 (Fig 4).

Discussion:

The salt test is currently the standard methodology to assess glochidial health, and uses a behavioral response (snapping shut) to estimate health in terms of viability (Fritts et al. 2014). Although viability, as estimated by the salt test, has been shown to be a valid proxy for infectivity (ability to attach to a host fish and successfully metamorphose to the juvenile stage) a major drawback is that it is applicable only to glochidia that are initially open. This is particularly problematic for studies involving cryopreservation in which otherwise healthy

glochidia may close in response to chemicals and freezing. Evaluation of cryoprotectant agents (CPA) and cryopreservation techniques requires assessment of glochidial health. However, the salt test cannot be used in cryopreservation studies because closed glochidia would be scored as nonviable even though they may be healthy and competent to metamorphose using *in vitro* (rearing in petri dishes and agar) rather than *in vivo* (rearing on a host fish) techniques. Similarly the use of the salt test in ecotoxicology assays may be limited because glochidia may close in response to chemicals, and thus be scored as nonviable, but unknown as to be live versus dead.

The dye assay represents a valuable alternative to the salt test. It was successful in distinguishing between live and dead glochidia regardless of whether or not the glochidia were initially open or closed. Importantly, the lethal endpoint (live) provided by the dye assay was not equivalent to the sublethal endpoint (viability) provided by the salt test. Thus it may be especially useful in ecotoxicology assays where the chemical stressor causes glochidia to close, but does not necessarily cause mortality. For example, when exposed to increasing concentrations of a reference toxicant that stimulated glochidia to snap shut, viability declined to a value of zero at NaCl concentrations $\geq$ 0.75 g/L. Conversely, the percent of glochidia scored as live decreased more slowly, not reaching a value of zero until a NaCl concentration of 6.75 g/L. This suggests that some glochidia that snapped shut in response to chemical exposure remained

alive. Use of viability as a proxy for lethal effects would have overestimated mortality caused by the reference toxicant.

Strong evidence suggested that the dye assay can be used as a proxy for sublethal effects such as infectivity. Because infectivity includes the ability to attach to fish gills, time was used as a stressor rather than a chemical stressor that would elicit a closing response. In the absence of a chemical stressor, the ability of the dye assay to predict metamorphosis success on host fish (*in vivo*) was nearly identical to that of the salt test, as evidenced be the nearly equal R^2 values of the two regression lines (Fig. 4). However, below values of 0.8, metamorphosis success declined more quickly with declines in percent live compared to declines in percent viability. Metamorphosis success had fallen to zero when >40% of glochidia were still scored as live. In contrast, metamorphosis success did not decline to zero until < 20% of glochidia were scored as viable. Thus, although not all live glochidia were able to metamorphose to the juvenile stage, the lethal endpoint (proportion of live glochidia) was as good a proxy for metamorphosis success as was the nonlethal endpoint (proportion viable).

The sodium chloride toxicity values from this study are comparable to NaCl toxicity test in other freshwater mussel species. In this study, according to the salt test, glochidia from *L. subrostrata* had a 4-h EC_{50}s ranging from 0.0 to 0.72 g/L NaCl and a 24-h EC_{50} of 0.0 g/L. The species in this study (*L. subrostrata*) seems to be more sensitive to NaCl compared to other

studies. Fritts et al. (2014) compared the NaCl toxicity of glochidia collected in June versus December from *Lampsilis cardium.* Glochidia from June had a 24-h EC_{50} of 0.80 ± 0.16 g/L while glochidia from Decemeber had a 24-h EC_{50} of 2.04 ± 0.11 g/L NaCl. Bringolf et al. (2007) conducted a study comparing five species of freshwater mussels and found that *Lampsilis siliquoidea* had the lowest EC_{50} (0.54 g/L) and *Villosa delumbis* had the highest EC_{50} (3.63 g/L). Different species of freshwater mussels are more/less sensitive to toxicants; *L. subrostrata* appears to be more sensitive in comparison to other species.

The advantage of the salt test is that it accounts for only open glochidia, which have the potential to attach to the fish host. Glochidia that are closed prior to exposure to salt are not considered viable because they lack the ability to re-open and then snap shut on a fish host, but could still potentially be transformed *in vitro*. The dye assay estimated the glochidial health to be better than the salt test; this is due to having the ability to evaluate the health of closed glochidia. Future studies should evaluate *in vitro* transformation and how the infectivity compares for the salt test and dye assay. The dye assay could be extremely valuable in terms of conservation. The application of cryopreservation to gametes and embryos has been used in marine bivalves to improve genetics, conservation, and seed production (Wang et al. 2014). Currently, cryopreservation of freshwater mussel glochidia has not been successful, and the dye

assay could be helpful in the development of a cryopreservation technique that could be useful in the conservation and restoration of endangered species.

Chapter 2 Evaluation of cryopreservation on glochidia using a fluorescent dye assay

Introduction:

Cryopreservation has been commonly used for the long-term preservation of gametes, embryos, or various tissues (Hanquet-Dufour 2006). Cryoprotectant agents (CPAs) are used to protect cells against damage caused during freezing via reducing ionic composition by increasing the unfrozen fraction at a given temperature. However CPAs are not universally beneficial and can cause both cytotoxicity and osmotically induced cell changes (Higgins 2014). There are two main types of CPAs: extracellular compounds (i.e. sucrose, trehalose, dextrose, and polyvinylpyrrolidone) that reduce hyperosmotic effect during the freezing process but do not penetrate the cell membrane and intracellular compounds (i.e. ethylene glycol, dimethyl sulfoxide, and glycerol) that penetrate inside the cell to prevent the formation of ice crystals leading to membrane rupture (Davis et al. 1990). CPA general properties are low molecular weight and relatively low toxicity (Moon et al. 2008; Ozkavukcu and Erdemli 2002). The toxicity of a CPA is dependent on concentration, duration, temperature, and developmental stage (Chao and Liao 2001).

Presently, the two methods of cryopreservation are slow-cooling and vitrification. Slow-cooling cryopreservation (used in this study) uses a lower CPA concentration and gradually decreases temperature before plunging specimens into -196°C liquid nitrogen (LN_2).

Intracellular ice can buildup and cause cell membranes to rupture during the subsequent thawing process. Slowly thawing of the cells can prevent this but can also cause dehydration from the formation of extracellular ice buildup. To prevent ice buildup, a CPA should be added before freezing and an ideal cooling rate should be established. The alternative is the vitrificaiton method, which uses a higher CPA concentration to prevent ice formation and specimens are placed directly into liquid nitrogen rather than being gradually cooled (Wang et al. 2014). Whittingham et al. (1972) were first in successfully cryopreserving embryos; they found mouse embryos survived freezing to -196°C and established that slow cooling (0.3-2.0°C/minute) and slow thawing (4-25°C/minute) methods had highest survival rates.

Many shellfish studies have focused on the cryopreservation of oyster eggs, sperm, and embryos. Embryos and trochophore larvae from *Crassostrea gigas*, *Crassostrea rhizophorae*, and *Crassostrea virginica* have successfully been cryopreserved (Gwo 1995; Nascimento et al. 2004; Paredes et al 2013; Paniagua-Chavez 2001; Paniagua-Chavez 2006; Tervit et al. 2005). Similar techniques were applied to trochophores, oöcytes, or sperm from the Greenshell mussel *Perna canaliculus* and the blue mussel *Mytilus galloprovinvialis,* but success rates were highly variable; very few D-larvae were produced (Adams 2009; Liu and Li 2015; Mullen et al. 2009; Paredes et al. 2012; Wang et al. 2014). The most commonly used CPAs in marine bivalve studies are ethylene glycol (EG), propylene glycol (PG) dimethyl sulfoxide (DMSO), methanol,

and 1,2-propanediol, plus the addition of one or more supplement (i.e. trehalose, fructose, galactose, glucose, sucrose, polyethylene glycol, and polyvinylpyrrolidone) (Wang et al. 2014). Liu et al. 2005 conducted a review on sperm cryopreservation in marine mollusks and found that DMSO promoted the highest metamorphosis success.

Currently, no known attempts have been made to apply cryopreservation techniques developed for marine bivalves to freshwater mussel glochidia. A literature search using Academic Search Premier and keywords (freshwater mussel; unionid; cryopreservation) yielded no papers. However, a general search using Google and keywords (freshwater mussel cryopreservation) found a March/April 2013 Activity Report from Warm Springs Fish Technology Center in which cryopreservation was attempted on *Ligumia subrostrata* but failed to transform *in vitro* (https://www.fws.gov/warmsprings/FishTechno/pdfs/FTC_Mar_Apr2013.pdf).

Assessment of glochidia is a major problem in the development of a successful cryopreservation technique for freshwater mussels. Glochidia need to be evaluated after each stage of cryopreservation to determine whether specific steps cause a drastic reduction in health or survival. However CPAs cause glochidia to close, eliminating the usefulness of the salt test, as well as *in vivo* metamorphosis of post-thawed glochidia. Fluorescein diacetate (FDA) and propidium idodide (PI) dye assay have been previously used in the assessment of cryopreserved

Campbell's hamster (*Phodopus* campelli) embryos and hard coral (*Echinopora spp.*) oöcytes (Amstislavsky et al. 2015; Tsai et al. 2010), and it is possible that a similar dual staining technique can be applied to glochidia.

Glochidia that have been cryopreserved are closed and do not have the ability to reopen, therefore glochidia cannot attach to a fish host. Rather, they must be transformed *in vitro*. *In vitro* transformation is an artificial propagation method that does not require a host for mussel larvae to metamorphose into juveniles, instead the larval cycle is completed in a Petri dish containing cell culture media, serum, and antibiotics (Lima et al. 2012). *In vitro* transformation has been successful in 42 species of unionoidids, several of which are federally endangered (Hudson and Shelbourne 1990; Isom and Hudson 1982; Keller and Zam 1990; Owen et al. 2010; Uthaiwan et al. 2001; Uthaiwan et al. 2002).

The objectives of this study were as follows:

1. Evaluate the effects of candidate cryopreservation agents on glochidial health and survivorship.
2. Determine if mortality during cryopreservation trials was primarily due to cryoprotectant agents or the freezing/thawing process.

Methods:

Evaluation of candidate CPA toxicity

Five candidate CPAs (1,2- propanediol, dimethyl sulfoxide, ethylene glycol, glycerol, and methanol) were tested for toxicity to glochidia at five concentrations (0%, 5%, 10%, 15%, and 20%) and four exposure times (5, 15, 25, and 35 minutes. A control (0% cryoprotectant) was used as a reference in all experiments. The goal was to find a CPA and concentration that caused less than 20% mortality during exposure. For each CPA tested, a stock solution was first prepared for each concentration by adding the appropriate volume of CPA to M199. A 20-ml aliquot of each stock solution was then pipetted into four 30-ml beakers (5 concentrations x 4 beakers/concentration = 20 beakers). Glochidia were extracted from gravid females and evaluated using the salt test. Broods from 3 females that exhibited >80% viability were mixed together in AFW and approximately 8,000 glochidia then transferred to each of the 20 experimental beakers. Beakers were held on a bench top at 22°C. At each of the four time periods, ten glochidia were removed from one of the four beakers associated with a given CPA concentration and gently swirled in 1 ml of M199 for ≥10 seconds to rinse off the cryoprotectant. The glochidia were then pipetted onto a depression slide (10 glochidia/slide) and stained with 50µl FDA and PI. Each slide was held in the dark for approximately 40 minutes and then scored (live:questionable:dead) under fluorescent microscopy. This procedure constituted a single "run"

for a given CPA. Four runs were conducted per CPA, yielding 4 replicates for each concentration X time combination (one replicate per run). New mussels were utilized for each run. Effect times (ETx = time to x% scored questionable or dead) and 95% confidence intervals (CI) around each ETx estimate were calculated using a Toxicity Relationship Analysis Program (TRAP 1.30, U.S.EPA, https://archive.epa.gov/med/med_archive_03/web/ html/trap.html) that fits a symmetric, sigmoidal effect versus exposure relationship to toxicity test data. TRAP analysis options were as follows: Analysis Type = Tolerance Distribution; Model Shape = Gaussian Distribution; Number of Parameters = 3; Effects Variable Transform = None; Exposure Variable Transform = None).

Evaluation of cryopreservation survival

On July 22, 2013 USFWS Warm Springs Technology Center in Warm Springs, GA, exposed freshly extracted *Ligumia subrostrata* glochidia to two concentrations of two cryoprotectants (5% methanol, 10% methanol, 5% dimethyl sulfoxide and 10% dimethyl sulfoxide). After exposure to the cryoprotectant for 18 minutes (2 replicates for each treatment), glochidia were transferred into plastic straws and placed in an automated KRYO 10 chamber for slow-cooling cryopreservation. In the KRYO 10 chamber, straws were held at 22°C for five minutes, then the temperature was reduced to -35°C at a rate of -2.5°C per minute and held for

ten minutes before being plunged in liquid nitrogen (-196°C) for 57 minutes and finally transferred to storage dewars. On April 21, 2014, samples were shipped and arrived at the South Auburn Research lab the next day. Straws of glochidia from each cryoprotectant treatment were thawed in a 35-45°C (95-113°F) water bath. Each straw was placed in the water bath for 9 seconds and to prevent contamination, the straw exterior was wiped off to remove any excess water before being emptied into a clean petri dish. Glochidia were then pipetted into a second petri dish containing Medium 199 (M199) to rinse off the cryoprotectant chemicals. Four subsamples were taken from each straw and placed on a depression slide. Each depression slide contained approximately ten glochidia and 50 µL of FDA and 50 µL of PI were applied to each depression. Slides were placed in the dark for a minimum of 20 minutes and glochidia were scored as live, questionable, or dead under fluorescent microscopy.

Results:

Evaluation of candidate CPA toxicity

Due to high mortality, neither ET_{20} nor ET_{50} could be estimated for any concentration of dimethyl sulfoxide. Proportion live declined to ≤ 0.2 within the first 5 minutes at all concentrations (Fig. 5). Methanol (5%) had an ET_{20} of 30 (22 – 37 CI) minutes and an ET_{50} of 45 (30 – 59 CI) minutes. Methanol (10%) had an ET_{20} of 28 (23 – 33) minutes and an ET_{50} of 36

(31 - 41) minutes. Methanol (15%) had an ET_{20} of 5 (0 – 12) minutes and an ET_{50} of 11 (7 - 15) minutes. Methanol (20%) had an ET_{20} of 1 (0 – 13) minute and an ET_{50} of 7 (0 – 14) (Fig. 6). Glochidia exhibited zero survival across all times for all glycerol concentrations (Fig. 7). Neither ET_{20} nor ET_{50} could be calculated for 1,2-propanediol due to high mortality. Proportion live declined to <0.4 within 5 minutes exposure to all concentrations (Fig. 8). Ethylene glycol (5%) had ET_{20} of 4 (0 – 44) minutes and an ET_{50} of 21 (10 – 41). Ethylene (10%) had an ET_{50} of 15 (0 – 43) minutes but ET_{20} could not be estimated due to high mortality (Fig. 9). Ethylene (15 and 20%) caused complete mortality across all exposure times.

Evaluation of cryopreservation survival

All thawed glochidia from all treatments (5% methanol, 10% methanol, 5% dimethyl sulfoxide, and 10% dimethyl sulfoxide) fluoresced bright red and were scored as "dead".

Discussion:

Assessment of glochidia is a major problem in the development of a successful cryopreservation technique for freshwater mussels. Mortality could occur during any stage of the cryopreservation process and evaluation after each stage would identify where alterations need to be made. According to Chao and Liao (2001) most cryoinjuries occur between 0 and -

40°C and are caused by heat removal or cryoprotectant application. The freezing process submerges glochidia into -196°C liquid nitrogen (LN_2) and the thawing process warms glochidia in approximately 9 seconds, therefore it is important to assess health after each process to track mortality. Glochidia close in response to CPAs and freezing/thawing, which makes the salt test unusable and there is no currently accepted technique to assess health of closed glochidia. The dye assay appears to be a valuable alternative based on evidence from Chapter 1. Fluorescein diacetate (FDA) and propidium idodide (PI) dye assay had been previously used in the assessment of cryopreserved Campbell's hamster (*Phodopus* campelli) embryos and hard coral (*Echinopora spp.*) oöcytes. Tsai et al. (2010) used the FDA and PI dye assay to determine methanol to be most toxic and ethylene glycol to be least toxic to hard coral oöcytes.

Researchers with the USFWS Fish Technology Center in Warm Springs, Alabama have been trying to develop cryopreservation techniques. A desired goal is to identify potential CPAs that cause less than 20% mortality after 18 minute exposure time. Based on our results, 5% and 10% methanol deserve further evaluation but dimethyl sulfoxide, glycerol, 1,2-propanadiol, and ethylene glycol do not. The results in this study determined that 5-10% methanol caused the least amount of damage to *L. subrostrata* glochidia and it had the greatest cryopreservation potential of the CPAs tested in this study. These results are similar to Kawamoto et al. (2007), who found success using 10% methanol to cryopreserve Japanese pearl oyster (*Pinctada fucata*

martensii) spermatozoa. In contrast to this study, other experiments specifically focusing on the cryopreservation of marine mussels and oysters found that methanol was most toxic to oyster oöcytes and ethylene glycol and dimethyl sulfoixde resulted in highest success/fertilization rates (Adams et al. 2009; Paredes et al. 2012; Paredes et al. 2013; Tervit et al. 2005; Wang et al. 2014). All but one study had the most success with 10% EG; Paredes et al. (2013) found that 15% was most effective for greenshell mussels. These contradictory results illustrate the need for species and stage-specific evaluations of CPA candidates. A CPA agent that works well for one species-stage combination may be toxic for others.

The dual staining technique scored all cryopreserved glochidia from previous trials at Warm Springs as "dead" after thawing, indicating subsequent *in vitro* transformation attempts were not warranted. These trials had utilized 18-minute exposures to 5-10% methanol and dimethyl sulfoxide as CPA agents.

Results of the previous CPA evaluation suggest that mortality most likely occurred during the exposure to the CPA for the dimethyl sulfoxide trials because no live glochidia were observed within 18 minutes of exposure to either 5 or 10% dimethyl sulfoxide. Conversely, survival after 18 minutes exposure to 5 and 10% methanol was still high, suggesting that death in the methanol cryopreservation trials more likely occurred during the freezing or thawing process. These results emphasize the value of the dye assay and suggest that future cryopreservation trials

should utilize methanol as a CPA and focus on optimization of the freezing and thawing processes.

USFW Warm Springs cryopreserved glochidia used in this experiment were via the slow-cooling method. A suggestion for future studies involving freshwater mussel glochidia cryopreservation would be to try the vitrification method as an alternative (Chao and Liao 2001; Want et al. 2014). Starfish oöcytes have successfully been cryopreserved using the vitrification method (Hamaratoglu et al. 2005). However, a drawback to vitrificaiton is it requires a higher CPA concentration. Adding an extracellular CPA or Co-CPA (i.e. trehalose) to reduce the hyperosmotic effect that occurs during the freezing process without penetrating the cell is also suggested. Many studies have found that the addition of 0.2 M trehalose increased fertilization and survival rates (Adams et al. 2009; Gale et al. 2014; Paredes et al. 2012; Wang et al. 2014).

Molluscs are considered structural engineers; filter feeding by freshwater mussels enhance benthos nutrient availability and mussel shells stabilize substrate and increase habitat heterogeneity (Atkinson et al. 2013). Freshwater mussels are North America's most imperiled faunal group and cryopreservation of freshwater mussels could be important for restoration and conservation of endangered species, as well as preserving genetic diversity and providing year-round glochidia. The ability to cryopreserve freshwater mussel would allow researchers to transform and grow-out preserved glochidia to introduce into declining or bottlenecked

populations. This could reduce the need to remove adult mussels from neighboring population for translocation purposes. Use of cryopreserved individuals would reduce the problems of depleting one population to restore another.

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Table 1. Results from infectivity experiment assessing the relative abilities of the salt test and dye assay to predict % metamorphosis success of extracted unionid glochidia.

Trial ID	Days post extraction	% Viable (Salt test)	% live (Dye assay)	% Metamorphosis success
1	0	90	95	74
2	0	87	90	79
2	1	80	84	77
1	4	75	79	45
2	3	71	87	43
2	6	66	72	42
1	6	64	70	41
2	9	50	66	27
1	7	49	63	35
1	8	39	52	41
1	11	23	49	18
2	10	13	43	0

Table 2. CPA concentration and volumes of components used in CPA toxicity experiment.

Concentration (%)	M199 (ml)	Cryoprotectant (ml)
0	20	0
5	19	1
10	18	2
15	17	3
20	16	4

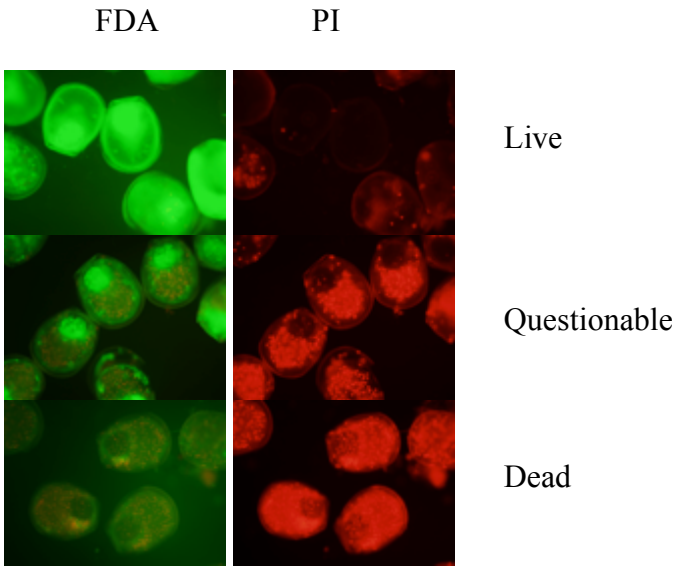

Figure 1. Representative glochidia scored as “Live”, “Questionable” or “Dead” after staining with FDA and PI for ~20 minutes. Glochidia were viewed through a compound, fluorescent microscope (Olympus DPBX41) at 10x magnification.

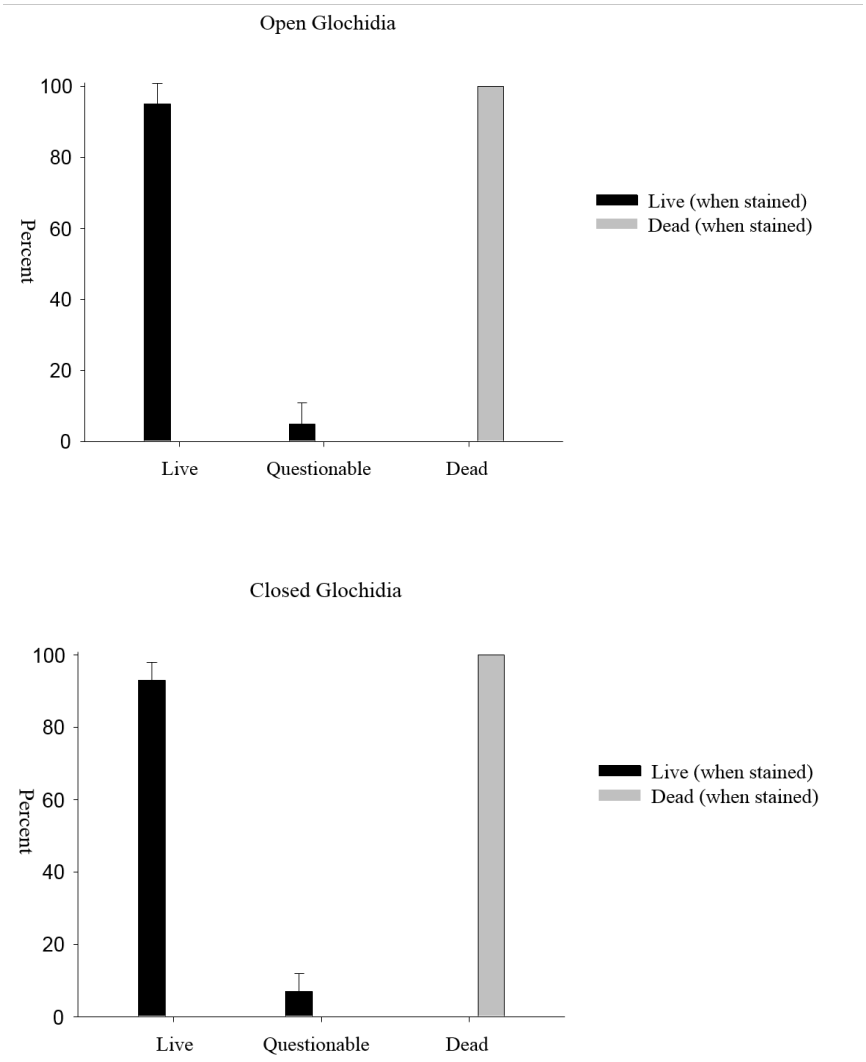

Figure 2. Percent of glochidia scored as Live, Questionable, or Dead after staining. Black bars refer to glochidia that were stained while live, grey bars refer to glochidia that were stained after freezing and thawing. The top panel shows results for glochidia that were stained while open. The bottom panel shows results for glochidia that were induced to close via exposure to salt prior to staining.

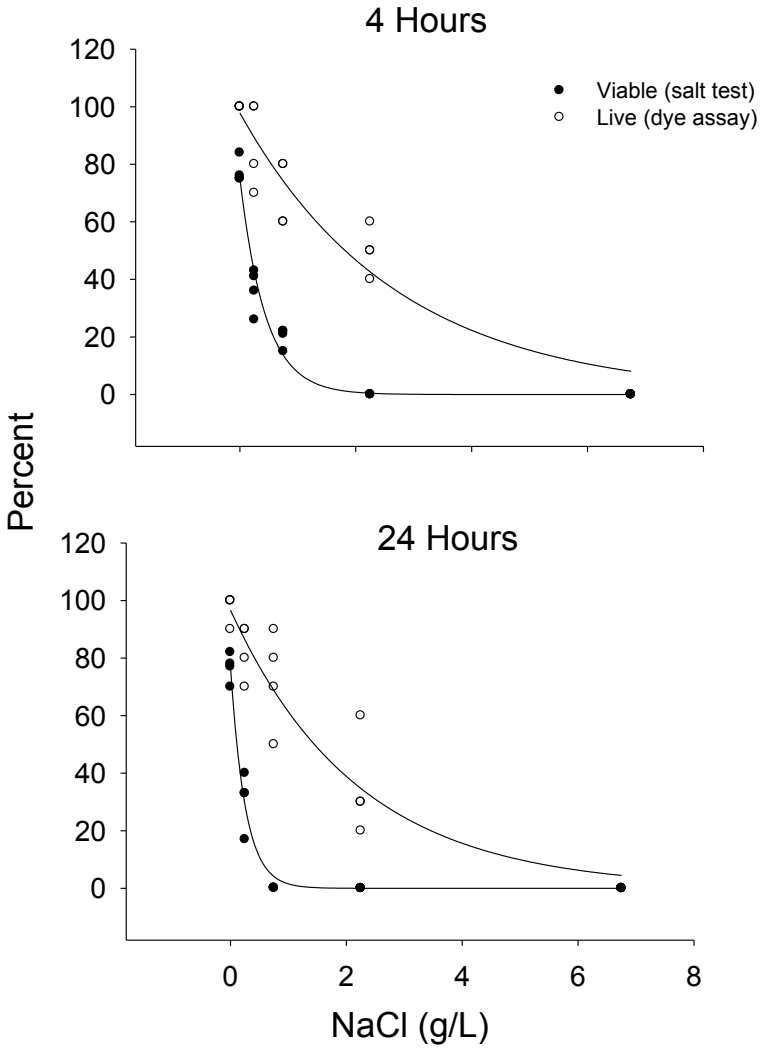

Figure 3. Relationship between percent glochidia scored as Viable (salt test) or Live (dye assay) relative to 4 hour (top panel) and 24 hour (bottom panel) exposure to increasing concentrations of NaCl.

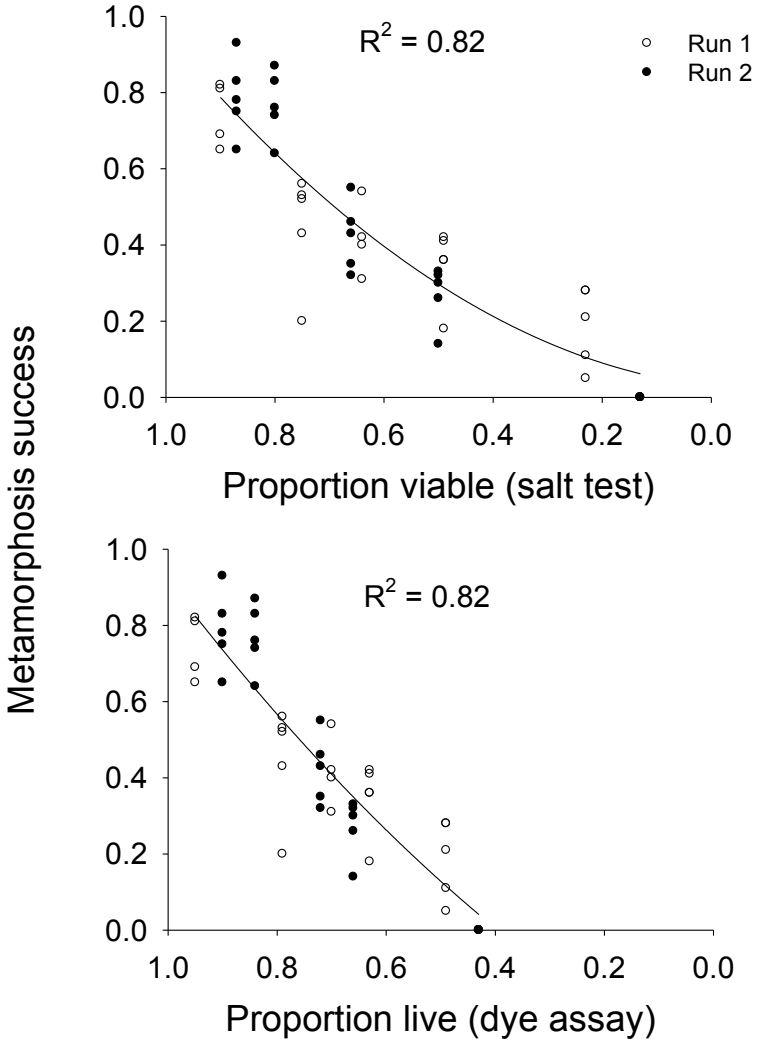

Figure 4. Relationships between metamorphosis success of mussel glochidia and relevant endpoints of the salt test (proportion viable) and dye assay (proportion live). Each point represents the metamorphosis success of glochidia encapsulated on a single fish. Open and closed circles represent fish inoculated with the first and second batches of glochidia, respectively. Lines represent quadratic regression through all data points.

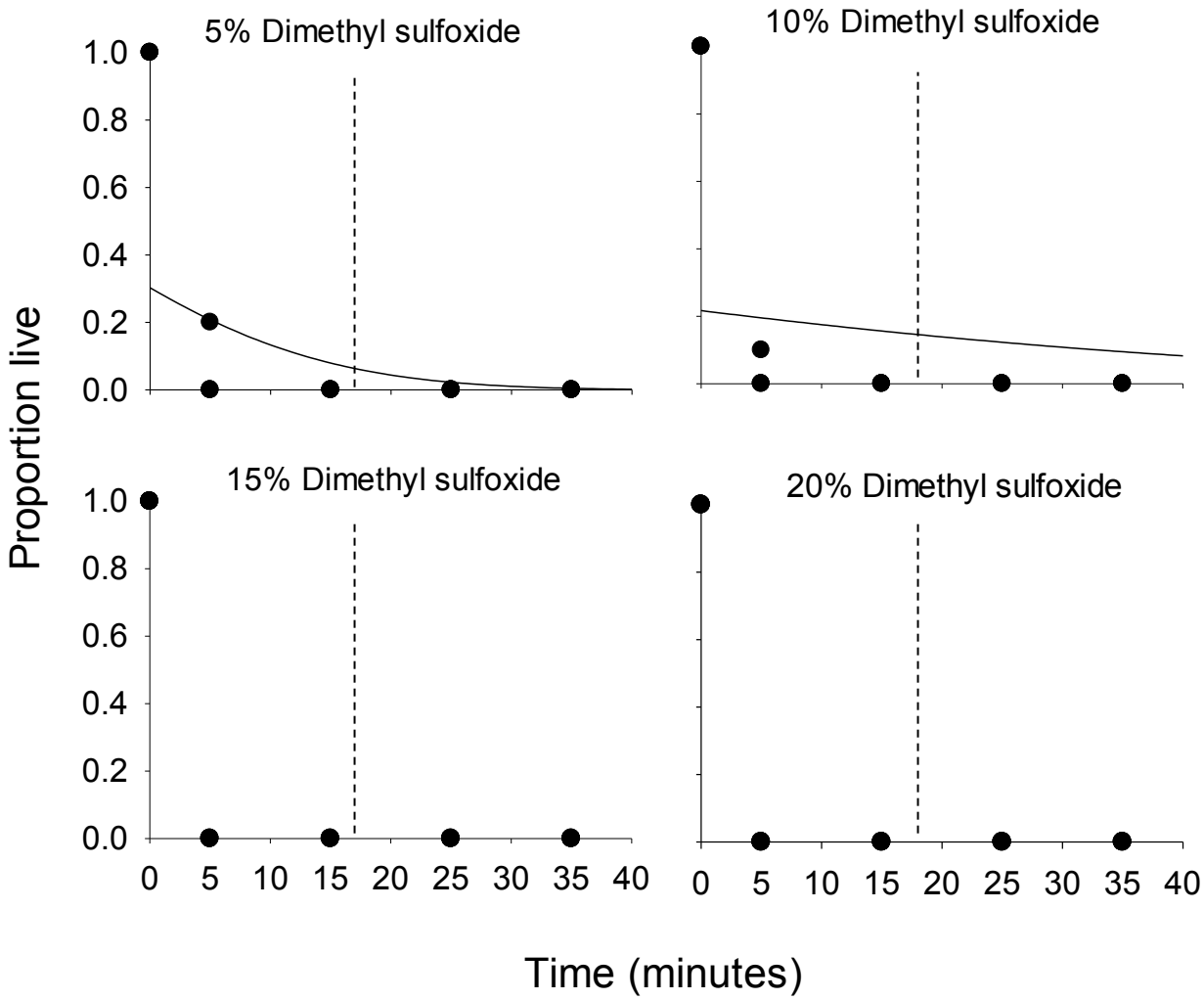

Figure 5. Relationships between proportion live and exposure time to Dimethyl sulfoxide. Solid lines represent sigmoidal regressions used by TRAP 1.3. to estimate ETx. Dotted lines mark the 18 minute CPA exposure time utilized by previous cryopreservation trial.

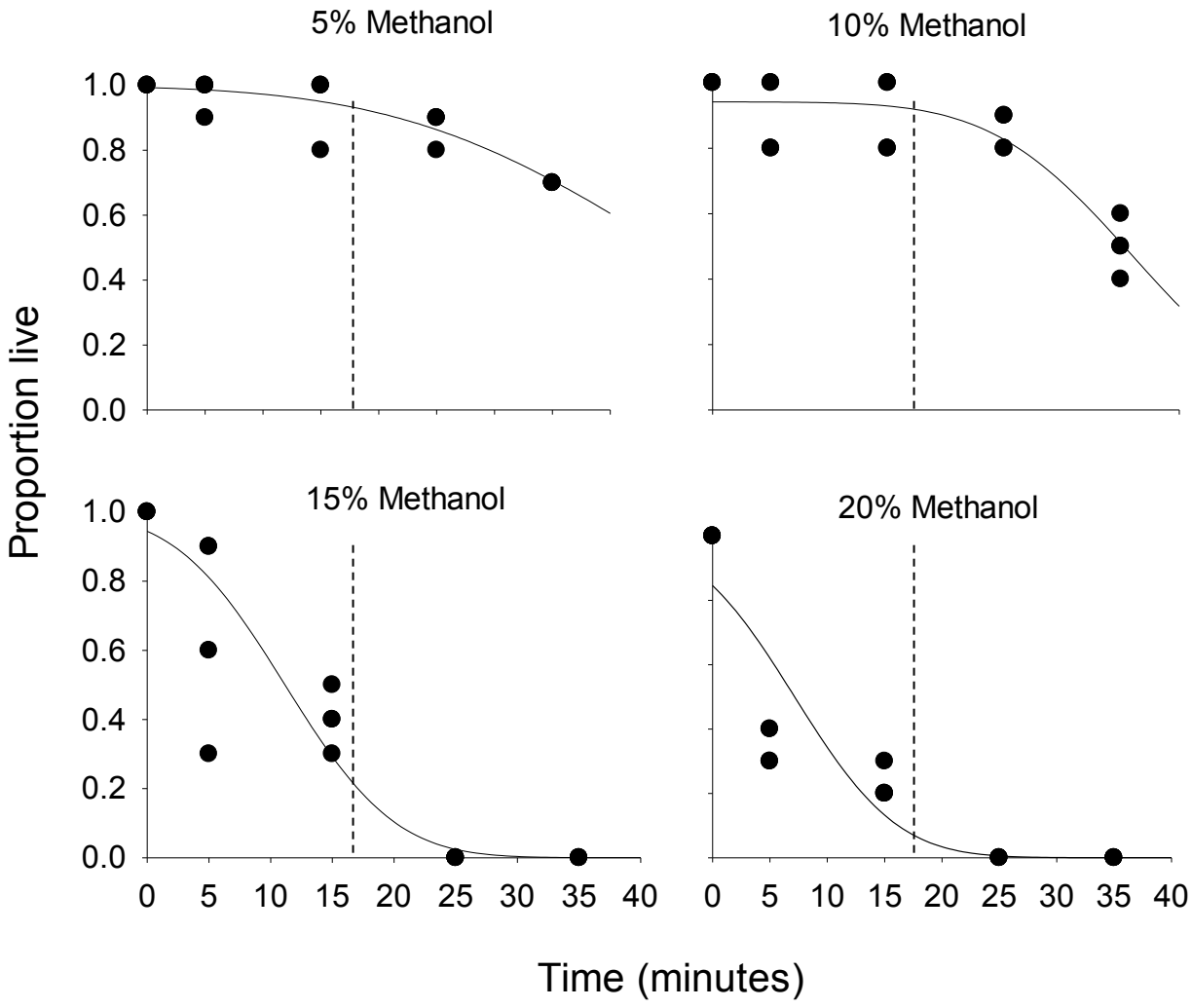

Figure 6. Relationships between proportion live and exposure time to Methanol. Solid lines represent sigmoidal regressions used by TRAP 1.3. to estimate ETx. Dotted lines mark the 18 minute CPA exposure time utilized by previous cryopreservation trial.

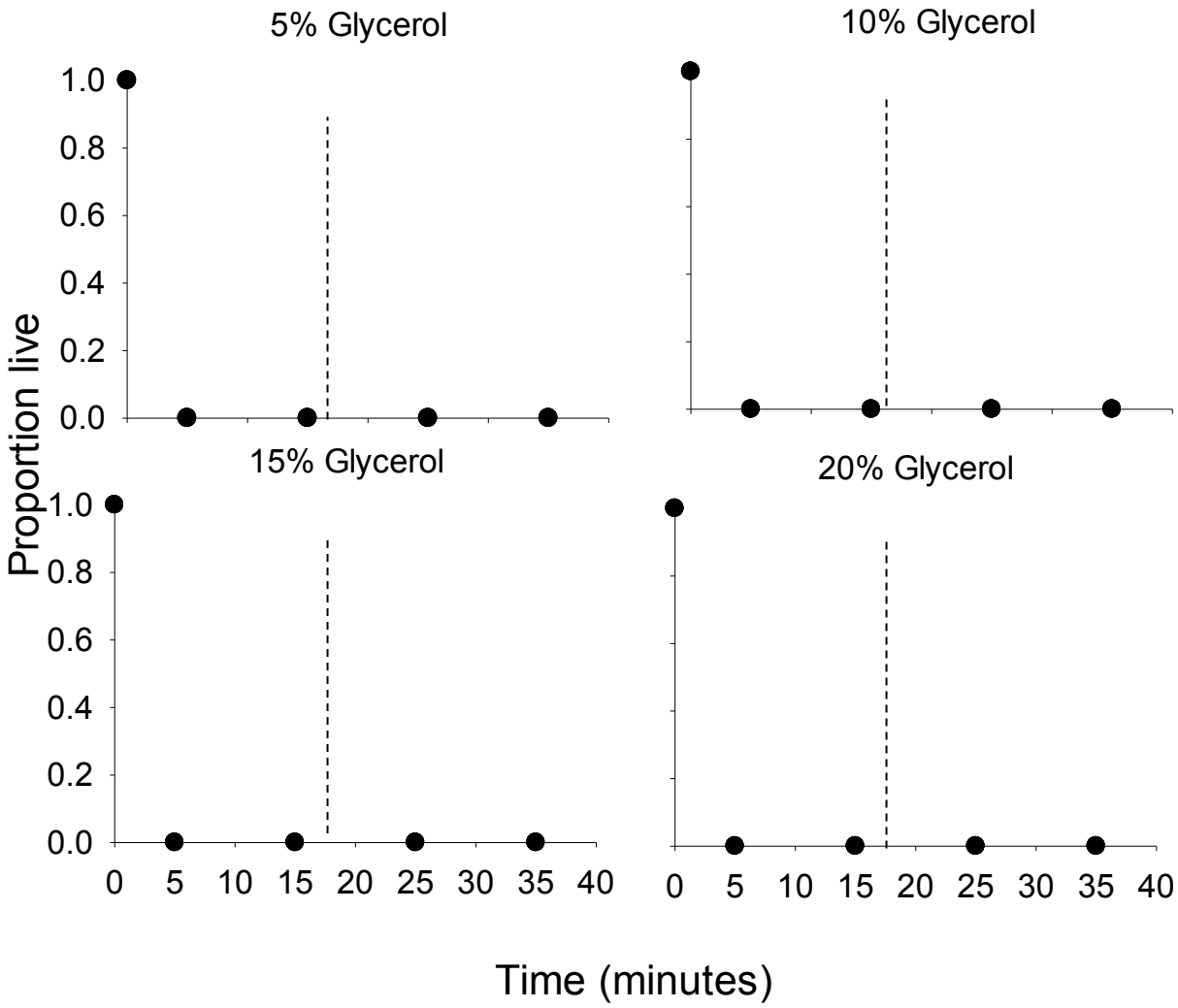

Figure 7. Relationships between proportion live and exposure time to Glycerol. Solid lines represent sigmoidal regressions used by TRAP 1.3. to estimate ETx. Dotted lines mark the 18 minute CPA exposure time utilized by previous cryopreservation trial.

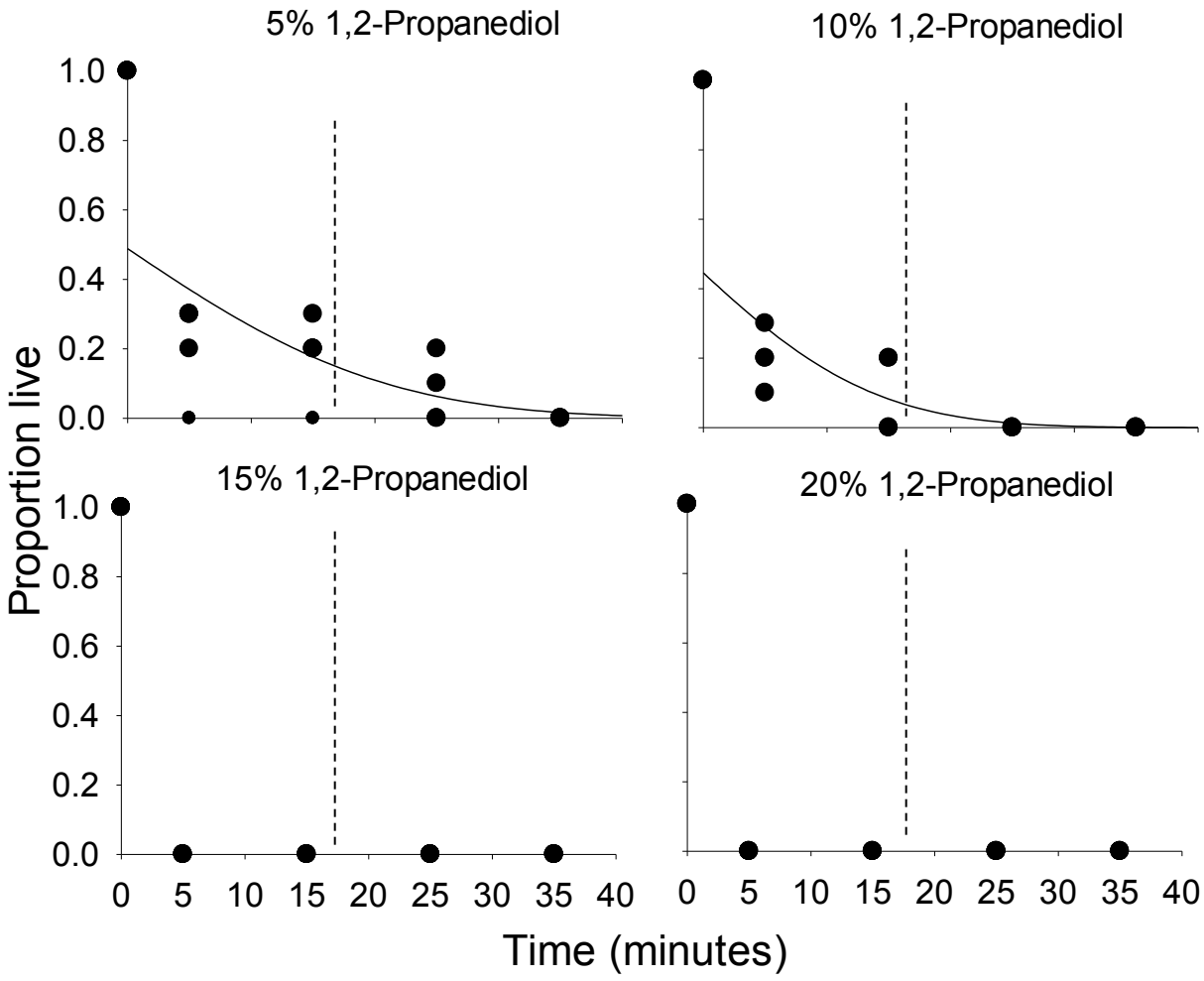

Figure 8. Relationships between proportion live and exposure time to 1,2-Propanediol. Solid lines represent sigmoidal regressions used by TRAP 1.3. to estimate ETx. Dotted lines mark the 18 minute CPA exposure time utilized by previous cryopreservation trial.

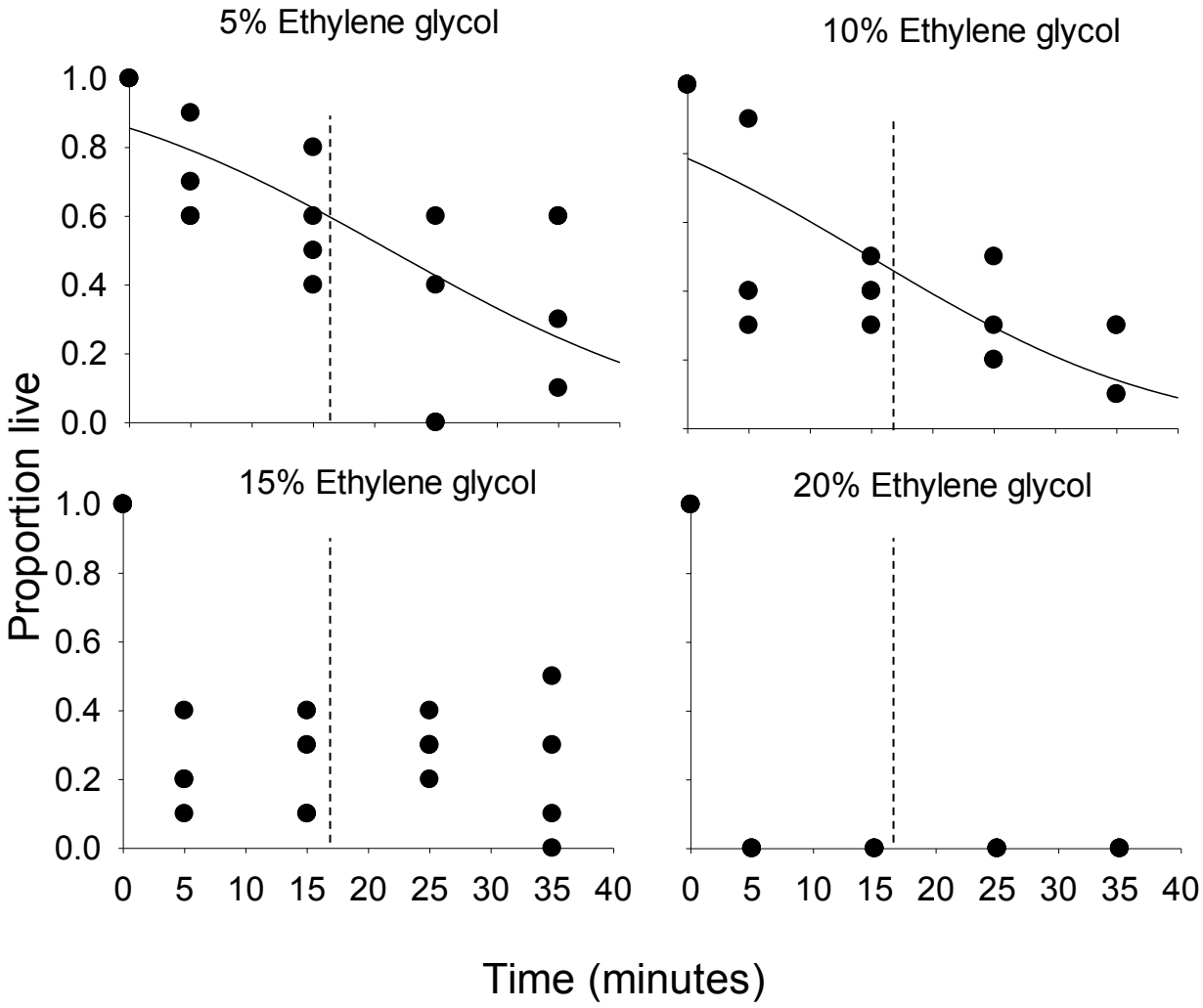

Figure 9. Relationships between proportion live and exposure time to Ethylene glycol. Solid lines represent sigmoidal regressions used by TRAP 1.3. to estimate ETx. Dotted lines mark the 18 minute CPA exposure time utilized by previous cryopreservation trial.